Targeting Motor Neuron - Immune System Crosstalk to Modulate the Disease Progression in Amyotrophic Lateral Sclerosis Mouse Model

Thesis

Targeting motor neuron - immune system crosstalk to modulate the disease progression in Amyotrophic Lateral Sclerosis mouse model

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ABSTRACT

ALS is a fatal neurodegenerative disease characterised by remarked heterogeneity, which might stem from the multisystemic, non-cell-autonomous and complex nature of the disease.

The early deterioration of the peripheral compartment has led to ALS being recognised as distal axonopathy, whereby muscles and nerves actively contribute to neurodegeneration. However, the contribution of the inflammatory response in the CNS starkly contrasts to the periphery, revealing its pivotal role at promoting phenomena of protection and/or toxicity.

We corroborated these observations showing a higher activation of the MCP1 chemokine within MNs and peripheral compartment of C57SOD1^{G93A} than 129SvSOD1^{G93A} mice. Therefore, we surmised that the higher peripheral degeneration and faster disease progression of 129SvSOD1^{G93A} mice stemmed from this defective immune response.

To decipher the contribution of the peripheral immune response in ALS progression, the therapeutic potential of MCP1 was assessed. The chemokine was induced alongside the motor units of the two SOD1^{G93A} models through a single intramuscular injection of a scAAV9 vector engineered with MCP1 (scAAV9_MCP1).

The scAAV9_MCP1-mediated boosting of the immune response prevented the degeneration of the peripheral compartment whilst the chemokine induction within MNs led to a neuroprotective activity, resulting in the amelioration of the clinical phenotype in C57SOD1^{G93A} but not 129SvSOD1^{G93A} mice.

This discrepancy pointed the nature and temporal activation of the immune response out as discriminating factors to promote the peripheral compartment regeneration and slow-down ALS progression.

The analysis of ALS patients muscles validated our findings, demonstrating a direct correlation between the immune cells inflammatory fingerprint and the rate of the disease progression.

These observations candidate the peripheral compartment as a primary target for the development of therapeutic interventions effective at influencing the ALS progression. Moreover, the

comprehension of the MCP1 role within the motor unit of SOD1^{G93A} mice might provide innovative evidence regarding the contribution of the immune response in ALS.

PREFACE

The work described herein was performed at the Istituto di Ricerche Farmacologiche "Mario Negri" - IRCCS, Milan, Italy, from October 2016 to October 2020.

The PhD research project was conducted under the supervision and direction of Dr Caterina Bendotti (Director of Studies) and Prof Andrea Malaspina (External Supervisor).

DECLARATION

This PhD research project has not been submitted in whole or in part for a degree or diploma or other qualification to any other University.

The experimental work described here was performed by me, Maria Chiara Trolese, and includes work carried out in collaboration with Dr Giovanni Nardo (Department of Neuroscience, Istituto di Ricerche Farmacologiche “Mario Negri” - IRCCS) who helped me with the immunohistological analysis and relative quantification.

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LIST OF ABBREVIATIONS

AChRγ	Acetylcholine Receptor gamma subunit
ALS	Amyotrophic Lateral Sclerosis
ALSFRS-R	Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised
Arg1	Arginase 1
BBB	Blood Brain Barrier
CCR2	C-C chemokine Receptor type 2
CD206	Cluster of Differentiation 206 (Mannose receptor)
CD4	Cluster of Differentiation 4
CD68	Cluster of Differentiation 68 (Macrosialin)
CD8	Cluster of Differentiation 8
ChAT	Choline Acetyltransferase
CNS	Central Nervous System
DAPI	4',6-diamidino-2-phenylindole
ER	Endoplasmic reticulum
fALS	familial Amyotrophic Lateral Sclerosis
FoxP3	Forkhead box P3
FTD	Frontotemporal Dementia
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GCM	*Gastrocnemius Caput Medialis*
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GM	*Gluteus Maximus*
gp91^{PHOX}/NOX2	Heme binding subunit of NADPH oxidase
hnRNP	Heterogeneous nuclear ribonucleoprotein
hSOD1	human Superoxide Dismutase 1
i.c.v.	intra-cerebro-ventricular
i.m.	intra-muscular
Iba1	Ionised calcium-binding adapter molecule 1
IGF1	Insulin-like Growth Factor 1
IL1β	Interleukin 1 beta

IL4	Interleukin 4
iNOS	inducible Nitric Oxide Synthase
LMN	Lower Motor Neuron
LPS	Lipopolysaccharide endotoxin
LVL	Left *Vastus Lateralis*
M1	classically activated microglia/macrophage
M2	alternatively activated microglia/macrophage
MBP	Myelin Basic Protein
MCP1/CCL2	Monocyte Chemoattractant Protein 1/ C-C motif chemokine Ligand 2
MN	Motor Neuron
MND	Motor Neuron Disease
MPC	Myogenic Progenitor Cells
mSOD1	mutant Superoxide Dismutase 1
MyoD	Myogenic Determination gene
MyoG	Myogenic factor 4
NCAM	Neural Cell Adhesion Molecule
NF200	Neurofilament heavy polypeptide
NMJ	Neuro-Muscular Junction
Ntg mice	Non-transgenic mice
O/N	Over Night
$p75^{NTR}$	p75 neurotrophin receptor
Pax7	Paired box 7
PCR	Polymerase Chain Reaction
PNS	Peripheral Nervous System
qRT-PCR	quantitative Real Time-Polymerase Chain Reaction
$RAG2^{-/-}$ mice	Recombination Activating 2 knock out mice
RBP	RNA binding protein
RQF	Right *Quadriceps Femoris*
RT	Room Temperature
sALS	sporadic Amyotrophic Lateral Sclerosis
SC	Schwann cell
scAAV9	self-complementary Adeno-Associated Virus serotype 9

SDH	Succinate Dehydrogenase
SEM	Standard Error of the Mean
SIRT1	NAD-dependent deacetylase Sirtuin 1
SOD1	Cu/Zn Superoxide Dismutase 1
T reg cells	T regulatory cells
TA	*Tibialis Anterior*
TB	*Triceps Brachii*
$TCR^{-/-}$	T cell Receptor knock out
Th1 cells	T helper 1 cells
Th2 cells	T helper 2 cells
TNFα	Tumour Necrosis Factor α
UMN	Upper Motor Neuron
UT	Untreated
WT	Wild type
ΔFS	deltaFS (ALS progression rate)

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INTRODUCTION

Chapter I

Amyotrophic Lateral Sclerosis (ALS)

1.1 AMYOTROPHIC LATERAL SCLEROSIS (ALS)

Amyotrophic lateral sclerosis (ALS) belongs to a broader group of disorders known as motor neuron diseases (MND), which are caused by gradual degeneration and death of motor neurons.

Motor neurons (MN) are a subclass of neurons present in the central nervous system (CNS) that extend from the brain to the spinal cord and muscles throughout the body. Motor neurons initiate and provide vital communication links between the brain and the voluntary muscles.

ALS is the most common MND. The first study of ALS date back to the mid-19th century when, in 1850, the English scientist Augustus Waller observed the appearance of shrivelled nerve fibres in cadavers. However, the first detailed description of ALS was made a few years later by the French neurobiologist and clinician Jean-Martin Charcot (Charcot and Joffroy, 1869). Charcot coined the name ALS, describing both the typical symptomatology and anatomopathological features of the disease. The term "Amyotrophic" refers to the muscle atrophy that characterises the disease; whereas "Lateral Sclerosis" describes the hardness of the lateral column of the spinal cord caused by the loss of upper motor neurons (UMN), axons that connect the brain with the lower motor neurons (LMN), which are replaced by activated glial cells.

In the 20th century, the baseball legend Lou Gehrig was diagnosed with the disease (1939). Due to his popularity in the United States and Canada, ALS is well known as "Lou Gehrig's disease".

Nowadays, ALS or Lou Gehrig's disease is classified as a rare progressive neurodegenerative disease caused by the loss of motor neurons of brain cortex (UMN), brainstem and spinal cord (LMN). The MN death causes the loss of the nerve impulse to voluntary muscles, which undergo progressive atrophy, eventually leading to the complete paralysis.

ALS is a poor prognosis disease, in which death usually occurs 3-5 years from the diagnosis due to the progressive denervation and dysfunction of respiratory muscles.

1.1.1 DIAGNOSIS

ALS is the most common form of MND, which also comprise progressive muscle atrophy (PMA) and primary lateral sclerosis (PLS) in which motor neurons loss is restricted to LMN and UMN,

respectively. ALS is characterised by the typical association of LMN and UMN degeneration, which produce the characteristic mixed picture. Due to the absence of a definitive and reliable test, the diagnosis of ALS relies predominately on the clinical evaluation, which is based on a history of progressive, painless weakness and examination findings of both LMN and UMN dysfunction. However, the symptom manifestation varies among patients depending on the subtype of neurons primarily affected (LMN or UMN) and the body regions involved. Because of the heterogeneity in the clinical manifestation, there are several "ALS-mimic syndromes" (Table 1). To avoid the misdiagnosis, a different diagnostic evaluation process, which includes electrophysiological studies and neuroimaging and biosamples (blood, cerebrospinal fluid, muscles biopsies) analysis, is applied to each patient in base on the first symptoms occurred.

Region/Involvement	UMN suspect findings	LMN suspect findings
Bulbar	Brainstem lesion (stroke, multiple sclerosis, tumour)	Brainstem lesion (stroke, multiple sclerosis, tumour), neuromuscular junction disorders (myasthenia gravis, muscle-specific tyrosine kinase myasthenia, bulbospinal muscular atrophy
Cervical	Cervical myelopathy	Multifocal motor neuropathy, cervical radiculopathy
Lumbosacral	Thoracic myelopathy	Lumbosacral radiculopathy, hereditary spastic paraparesis

Table 1: *Diseases commonly considered in the differential diagnosis of ALS (Modified from Oskarsson et al., 2018).*

Formal criteria for the diagnosis of ALS have been defined by the World Federation of Neurology at the meeting in El Escorial (Spain) in 1994 (Brooks 1994). The EEC (El Escorial criteria) illustrate four different levels of diagnosis depending on the subtype of MN and the body region (bulbar, cervical, thoracic and lumbosacral) affected: certainty, namely definite, probable, possible or suspected.

In 1998, in Airlie House (Warrenton, VA, US) an experienced group of clinicians revised the EEC adding a level of certainty "probably ALS-laboratory supported", defined after the proper application of clinical laboratory protocols and neuroimaging. Besides, the "suspected ALS" level was removed (Brooks et al., 2000; Oliveira and Pereira 2009) (Table 2).

LEVEL of CERTAINTY	CLINICAL MANIFESTATION
DEFINITE ALS	✓ UMN and LMN signs in the bulbar region and at least two spinal regions, or ✓ UMN signs in two spinal regions and LMN signs in three spinal regions.
PROBABLE ALS	✓ UMN and LMN signs in at least two regions, with some UMN signs rostral to LMN signs
PROBABLE LABORATORY-SUPPORTED ALS	✓ Clinical evidence of UMN or LMN signs in only one region; or ✓ UMN signs alone in one region and LMN signs defined by EMG criteria in at least two muscles of different root and neve origin in two limbs.
POSSIBLE ALS	✓ UMN and LMN in only one region; or ✓ UMN signs in two or more regions; or ✓ LMN signs rostral to UMN signs.

Table 2: *Revised El Escorial criteria (EEC) for the ALS level classification.*

The EEC have been criticised for being overly restrictive in the usage of electrophysiology data and for being insensitive to ALS diagnosis based on convention clinical evaluation. Indeed, in ~10% of cases, even at the death, the EEC-based diagnosis is categorised as “possible”, and only ~31% of patients meet the criteria of “define ALS” at the time of diagnosis (Traynor et al., 2000; de Carvalho and Swash 2011).

In 2006, the Awaji-shima (Japan) criteria simplified the ECC classifying the certainty level of diagnosis into one of three categories: clinically definite, probable and possible (Table 3). The Awaji-criteria, aligning the importance of electrophysiology to the clinical observation, were designed for daily clinical practice and early diagnosis; conversely, the EEC seemed to be more useful for researchers and clinical trials enrolment (de Carvalho and Swash 2011; Silani et al., 2011).

REQUISITE FOR DIAGNOSIS	✓ Presence of evidence of LMN degeneration by clinical, electrophysiological or neuropathological examination; ✓ Presence of evidence of UMN degeneration by clinical examination; ✓ Presence of progressive spread of symptoms or signs within a region or to other regions, as determined by history, physical examination or electrophysiological tests; ✓ Absence of electrophysiological or pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration; ✓ Absence of neuroimaging evidence of other disease processes that might demonstrate the observed clinical and electrophysiological signs.

DIAGNOSTIC CATEGORIES	✓ *Definite ALS*: clinical or electrophysiological evidence by the presence of LMN as well as UMN signs in the bulbar region and at least two spinal regions or the company of LMN or UMN signs in three spinal regions; ✓ *Probable ALS*: clinical or electrophysiological evidence by the presence of LMN and UMN signs in at least two regions with some UMN signs necessarily rostral to (above) the LMN signs; ✓ *Possible ALS*: clinical or electrophysiological signs of UMN and LMN dysfunction in only one region or UMN signs alone in two or more regions or LMN rostral to UMN signs.

Table 3: *ALS criteria according to Arlie House criteria in light of Awaji-shima consensus recommendations (Modified from Lenglet and Camdessanché, 2017).*

Although the clinical examination let the ALS diagnosis reasonably straightforward, the main challenges remain: *i*) the considerable time elapses between the appearance of the first symptoms and the reaching of the definite diagnosis (Palese et al., 2019); *ii*) the conspicuous number of false-negative (26-42%)/-positive (8-10%) (Chiò 2000); *iii*) the misdiagnosis due to the "ALS-mimic syndromes" (Quarracino et al., 2019) and *iv*) the heterogeneity in symptoms manifestation and speed of disease progression (Ticozzi and Silani 2018, Bendotti et al., 2020).

1.1.2 SYMPTOMATOLOGY

ALS symptoms are related to the dysfunction and loss of UMN and LMN. The majority (~75%) of ALS patients develop a limb-onset, while ~25% of patients exhibit a bulbar-onset, according to the body region firstly affected by the disease (extremities *versus* throat and mouth muscles, respectively). Only ~5% of subjects present initial trunk or respiratory involvement, subsequently spreading to other body regions (Kiernan et al., 2011).

The most common symptoms of ALS are fatigue and reduced exercise capability that force patients to need assistance *in continuum*. Nonetheless, the presentation can vary depending on the UMN or LMN involvement, which define the symptoms related to the bulbar or limb-onset (Kiernan et al., 2011) (Table 4).

	UMN	LMN
BULBAR ONSET	✓ Spastic dysarthria	✓ Tongue wasting, weakness and fasciculation; ✓ Flaccid dysarthria; ✓ Dysphagia.

LIMB ONSET	✓ Weakness; ✓ Lack of coordination; ✓ Rigidity; ✓ Spasticity; ✓ Increased tendon reflex; ✓ Extensor plantar responses.	✓ Fasciculation; ✓ Upper and lower limb wasting; ✓ Weakness

Table 4: *ALS related symptoms depending on the type of onset and motor neuron subtype involvement.*

As discussed above, ALS is characterised by a higher heterogeneity in terms of clinical manifestation and speed of the disease progression, confounding factors that complicate the process of diagnosis (Talbot 2009). The rate of functional decline, progression and survival might be related to the initial clinical manifestation. Nevertheless, studies performed in disease animal models suggest a delay between the MN damage and the appearance of symptoms (Kennel et al., 1996).

The variableness of symptoms decreases as the disease progresses, conforming in muscular wasting and paralysis, eventually leading to a bedridden state. As the disease progresses, also respiratory muscles are affected impairing the respiratory activity, that constrains patients to mechanic ventilation. Respiratory failure is the principal cause of death in ALS patients without tracheostomy (Salameh et al., 2015).

In the second half of the XX century the commonly used instrument for the assessment of the disease status and progression in ALS included i) the Norris scale, ii) the Baylor (Appel) scale and iii) the Tufts Quantitative Neuromuscular Examination (Cedarbaum and Stambler 1997). However, these instruments were not very operational.

In the same years, the robustness and consistency of the ALS Functional Rating Scale (ALSFRS) was demonstrated in multicentre clinical trial ALS CTNF treatment study (Cedarbaum and Stambler 1997). Developed in 1996 by the World Federation of Neurology (Group ACTSAPI-IS 1996), the ALSFRS is an ordinary rating scale consisting of 10 sets of 5 questions scored from 0 to 4, where 4 is a normal function. These domains comprise 3 bulbar sets (speech, salivation and swallowing), 6 motor sets (3 upper and 3 lower limbs) and 1 breathing set used to evaluate the status of patients. A few years later, the ALSFRS was revised (ALSFRS-R) adding 3 additional respiratory sets, addressing dyspnoea, orthopnoea and use of mechanical respiratory aids (Cedarbaum et al., 1999).

However, measurement of change, in the absence of reliable biological markers, remains an elusive clinical exercise.

Notwithstanding the heterogeneity in symptoms manifestation, ALS is a poor prognosis disease. About 50% of patients die within 30 months of the first clinical manifestation, and ~20% of patients survive between 5-10 years from the diagnosis (Chiò et al., 2009). Moreover, a simple prognostic algorithm based on a multivariate model indicated an association between upper limb or bulbar weakness, executive dysfunction and ALSFRS-R slope before first evaluation as negative prognostic indicators (Elamin et al., 2015). The biological basis of such differences is not understood. Even in families with specific gene mutations, affected members may manifest clinical heterogeneity supporting the likelihood that there are gene modifiers and pathways that specifically govern the disease manifestation (Camu et al., 1999).

Besides "pure" motor symptoms, a multifaceted degree of extra-motor involvement (e.g. lexical fluency deficit, impaired language and associativity judgement, social cognition disability) has been observed in ALS patients that kindled the attention in the overlap between ALS and frontotemporal dementia (FTD) (Christidi et al., 2018).

1.1.3 NEUROPATHOLOGY

Structural and histological studies performed on *post mortem* tissues of ALS patients permitted to increase the comprehension of this disease.

Starting from the XIX century, a variety of clinical descriptions of the disease were made. This effort culminated in the correlation between the key clinical features of progressive muscle atrophy and spasticity and the key neuropathological features described by Charcot, such as the loss of anterior horn cells and sclerosis in the lateral columns.

Subsequent studies contributed to a more in-depth characterisation of ALS neuropathology, including i) the observation of the loss of giants cells of Betz (Hammer et al., 1979; Nihei et al., 1993); ii) the identification of eosinophilic inclusion called "Bunina bodies" (Okamoto 1993); iii) the discovery of ubiquitinated cytoplasmic inclusions (Leigh et al., 1988; Lowe et al., 1988) and the identification of their main constituents (i.e. TDP-43) (Arai et al., 2006; Neumann et al., 2006), and,

in the last few years, iii) the association between the ALS and FTD neuropathology (Hudson 1981; Kiernan and Hudson 1994).

Macroscopically, no gross alteration has been found in most brain with ALS, albeit some analyses reported atrophy of the precentral gyrus (Qin et al., 2018). Frontal or temporal cortex atrophy was found most significant in the brain of patients with overlap ALS-FTD (Mioshi et al., 2013). The spinal cord shows atrophy of anterior nerve roots (Chang et al., 2005; Murphy et al., 2007). In addition to the grey matter, a reduction of the white matter was observed, particularly in the corticospinal tract (Roccatagliata et al., 2009; Zhang et al., 2014b).

The typical microscopic hallmarks of ALS are the loss of large MN (but not just large neurons) in the anterior horn of the spinal cord and lower cranial motor nuclei of the brainstem and Betz cells in the V layer of the primary motor cortex (Hammer et al., 1979; Nihei et al., 1993). Furthermore, it has been reported clear evidence of reduction of neuron size as well as atrophy and loss of myelinated axons (Tandan and Bradley 1985). Other pathological features of ALS include vacuolisation, vast empty spaces near neurons, spongiosis and the presence of Bunina bodies mostly within MN and occasionally in dendrites (Piao et al., 2006; Tomonaga et al., 1978; Kuroda et al., 1999). Unaccountably, some LMN are spared by the disease: Onufrowicz nucleus (located in the S2 spinal segment) and the cranial MN, which govern the pelvic floor musculature and the extraocular muscles permitting the maintenance of the faecal and urinary continence and the ocular movement respectively (Iwata and Hirano 1978; Wijesekera and Leigh 2009).

ALS is the most common MND. The typical hallmarks of the MN pathology are:

- ✓ *Cytoplasmic inclusions*_ There are three different subtypes of cytoplasmic inclusions: skin-like, Lewy-body and hyalin conglomerates inclusions (HCI). The skin-like inclusions are specific for ALS, while Lewy-body and HCI are present in other neurodegenerative diseases (Jellinger 2008; Leigh et al., 1989). These inclusions are immune-positive for ubiquitin, a protein “tag” necessary for the degradation of misfolded or senescent proteins (Bendotti et al., 2012), and composed of neurofilaments associated with proteins and organelles and the nuclear factor TAR-DNA binding protein 43 (TDP-43) (Neumann et al., 2006). The only

ubiquitin and TDP43-negative inclusion is the Bunina body, which is composed by eosinophilic aggregates (Okamoto et al., 2008).

- ✓ *Mitochondrial modifications*_ Morphologically, these organelles appear swelled, vacuolated and with a dense conglomerate of aggregates. Moreover, alterations have been found in respiratory chain enzymes and programmed cell death (Ruffoli et al., 2015; Martin 2011).
- ✓ *Golgi apparatus fragmentation*_ Morphologically the organelles appear smaller, disconnected and more numerous (fragmented) (Mourelatos et al., 1993; Stieber et al., 1998). These alterations are accompanied by loss/gain of function in protein sorting, processing and transport along the axons. Moreover, the organelles fragmentation allows the activation of pro-apoptotic pathways that contributes to MN loss (Haase and Rabouille 2015).
- ✓ *Axonal cytoskeleton dysregulation*_ *Post mortem* studies showed the presence of axonal spheroids and perikaryal accumulations/aggregations comprised of the neuronal intermediate filament proteins, neurofilaments and peripherin that impairs the axonal transport (Julien 1995; Xiao et al., 2006). In the last decades, the dosage of neurofilaments (phosphorylated and the light or heavy chain) in biofluids is a prevalent diagnostic and prognostic tool (Poesen and Van Damme 2018; Benatar et al., 2019).

More recently, several studies performed in patients and disease models have highlighted the crucial role of glial cells in the biology of ALS neurodegeneration, showing reactive astrogliosis surrounding degenerating MNs (Boillée et al., 2006b; Lasiene and Yamanaka, 2011). Astrocytic activation is notable in the grey matter of the ventral horn of the spinal cord, which is accompanied by hyaline inclusions and oxidative and nitrative stress markers (Philips and Rothstein 2014). Also, microglia activation represents a critical aspect of ALS neuropathology. Indeed, once activated, microglia responds to the neuronal distress releasing a plethora of pro-inflammatory factors heightening the phlogosis (Philips and Rothstein 2014). Moreover, the degree of microglial

activation is correlated to the severity of UMN degeneration (Turner et al., 2004; Lasiene and Yamanaka 2011).

However, ALS is a multifactorial and multisystemic disease due to the severe alteration observed in multiple tissues and body compartments, including nerves and skeletal muscles (Wijesekera and Leigh 2009). These observations led to defining ALS as a "non-cell autonomous" disease (Ilieva et al., 2009).

The damage of peripheral nervous system (PNS) is an early event in ALS pathogenesis, anticipating MN degeneration and motor function decline (Dadon-Nachum et al., 2011), and represents a major determinant of patients disability (Riva et al., 2016; Gentile et al., 2019). Nowadays, the nerve biopsy analysis is a procedure propaedeutic to the diagnosis (Riva et al., 2011). Moreover, preclinical studies showed a positive correlation between the extent of PNS damage and the ALS-related mutation or the speed of disease progression (Nardo et al., 2016b; Tian et al., 2016).

As proof of the "die-back" phenomenon that characterises ALS pathology (Dadon-Nachum et al., 2011), also the alteration of skeletal muscles is an early event in the disease. Indeed, muscle weakness is the pivotal sign of the disease in both patients and models (particularly mutant SOD1 mice). Several studies showed that ALS muscles suffer from oxidative stress, mitochondrial dysfunction and bioenergetic disturbance. However, the implication of muscles in nourishing the degenerative process is still under debate (Loeffler et al., 2016). Furthermore, the knowledge of the processes underlying the degeneration/regeneration mechanisms and the myogenic potential of ALS muscles is still limited (Jensen et al., 2016). Indeed, studies are still ongoing to clarify the different susceptibility of the muscular compartment to the disease (Nijssen et al., 2017; Di Pietro et al., 2018; Jensen et al. 2016).

In the last years, growing attention has been focused on events related to the innate and adaptive immune response in ALS determination and progression. Several studies demonstrated that ALS patients also show abnormalities in the circulating blood cells (Mantovani et al., 2009; Gustafson et al., 2017). In particular, it has been described deregulation in levels or expression profile of dendritic cells (Rusconi et al., 2017), monocytes (Zondler et al., 2016; Zhang et al., 2006) and T lymphocytes

(Katchar et al., 2001; Zhang et al., 2005). These studies showed that these alterations might be the mirror of the pathological processes within CNS and put them forward as predictors of disease progression (Murdock et al., 2016; Nardo et al., 2011; Rahman et al., 2019; Swindell et al., 2019).

1.1.4 EPIDEMIOLOGY

Amyotrophic lateral sclerosis epidemiology has rapidly developed in the last 30 years alongside the evolving changes in concepts in the field of clinical ALS and due to the recent proposals of a new classification system for motor neuron diseases (Al-Chalabi et al., 2016).

ALS is considered a rare disease with an incidence (number of new cases per year) between 0.6 and 3.8 per 100˙000 person-year (p-y). In Europe ALS incidence is higher (ranging from 2.1 to 3.8 per 100˙000 p-y), in contrast other population-based studies have measured the lowest incidence in East and South Asia (0.89 and 0.79 per 100˙000 p-y, respectively) (Longinetti and Fang 2019; Logroscino and Piccininni 2019). Speculations for a lower incidence registered in Asia are the absence of population-based studies (the first registry in Europe was established in Scotland in 1989 (Hern et al. 1992)) and the lower prevalence of ALS-associated genes in the Asian population (Kim et al., 2016). The origin of the geographic incidence of ALS is a matter of debate, since it is partly due to the prevalence of ALS-associated genes and partly to the environmental risk factors. Another confounding factor in establishing the incidence of ALS might derive from the delay in the diagnosis. Indeed, although the closer surveillance of patients with familial ALS led to early diagnosis, the type of onset and the heterogeneous clinical manifestation can postpone it. Patients with bulbar onset were reported to be diagnosed earlier compared to them with spinal onset. Moreover, male patients were reported to be diagnosed on average sooner than females (Longinetti and Fang 2019).

Recent population-based studies reported a prevalence between 4.1 and 8.4 per 100˙000 person (Longinetti and Fang 2019). A difference in ALS prevalence by ethnicity has also been recently reported. Using the National ALS Registry the prevalence of European- American ALS patients was found to be more than double the prevalence of African-American ALS patients (5.4 versus 2.3 per

100˙000) (Mehta et al., 2018). Furthermore, a male to female ratio between 1 and 2 was reported, except for Africa, where this ratio was registered higher than 2.9 (Longinetti and Fang 2019).

ALS is considered a disease of the adult because the peak of onset is between 51 and 66 years. European patients usually have a later age onset compared to Asia and Latin America (Longinetti and Fang 2019).

Despite the predominance of spinal onset (58-82%) in all countries, bulbar onset seems to be prevalent in subjects characterised by different traits (females, cognitive impairment, elderly, etc.). In addition to the spinal or bulbar onset, recently have been reported other types of onset that might alter the incidence of the disease in ALS registers: mixed (spinal and bulbar), thoracic, cognitive and respiratory (Longinetti and Fang 2019).

1.1.5 GENETICS

ALS is considered a multifactorial disease due to an interplay between environmental and genetic factors as disease determinants. The disorder exists in sporadic and familial forms uniformly throughout the world, except for a higher familial incidence in Guam island and Kii peninsula of Japan (Kuzuhara et al., 2001). The majority of cases (~90%) are sporadic (sALS) with no apparent genetic linkage. In comparison, in ~5-10% of the patients, the pathology is familial (fALS) and caused by the inheritance of a specific mutation (Ajroud-driss and Siddique 2015). Generally, in adult-onset ALS, the disease is inherited as an autosomal dominant (AD) trait. However, rare cases of juvenile ALS are commonly associated with autosomal recessive (AR) or X-linked inheritance. Intriguingly, it has been reported an AR inheritance of AD genes in specific populations (e.g. *FUS* in Cape Verde and *SOD1* in Scandinavia) (Alsultan et al., 2016).

Several factors may contribute to the missing hereditability in ALS, including the complex nature of the disease and the limitations of the technologies used in large association studies. These studies are based on short read and high throughput technologies (van Rheenen et al., 2016), which, albeit useful in the detection of single-nucleotide polymorphisms (SNP), are not able to identify the majority of structural variations of the human genome (e.g. long repeats, repetitions in multiple DNA regions, etc..) (Naruse et al., 2019).

Nonetheless, the clinical phenotype of sALS and fALS patients is usually indistinguishable, even though fALS cases exhibit an earlier onset (~46 years) compared to sALS (~56 years) (Camu et al., 1999). Inexplicably, in sALS a male preponderance was reported (1.5:1) respect to fALS (1:1), although this ratio tends to decrease after age 70 (Haverkamp et al., 1995; Gros-Louis et al., 2006; Naruse et al., 2019).

Familial ALS (fALS)

Each newly discovered gene implicated in the aetiology of ALS provides fundamental insights into the pathogenesis of MN degeneration of this disease, as well as facilitating models generation and, thus, the preclinical testing of new therapeutic interventions.

All genes implicated in fALS so far identified and the respective ALS subtype are listed in the table below.

Inheritance	*Denomination*	*Locus*	*Gene*	*Protein*	*Reference*
Autosomal Dominant	ALS 1	21q22.11	*SOD1*	Cu/Zn superoxide dismutase 1	(Rosen 1993)
	ALS 3	18q21	*unknown*	unknown	(Hand et al. 2002)
	ALS 4	9q34.13	*SETX*	Senataxin	(Chen et al. 2004)
	ALS 6	16q11.2	*FUS/TLS*	Fused in sarcoma/translated in liposarcoma	(Ruddy et al. 2003; Kwiatkowski et al. 2009; Vance et al. 2009)
	ALS 7	20p13	*unknown*	unknown	(Sapp et al. 2003)
	ALS 8	20q13.32	*VAPB*	Vescicle-associated membrane protein B & C	(Nishimura et al. 2004)
	ALS 9	14q11.2	*ANG*	Angiogenin	(Greenway et al. 2004 and 2006; van Es et al. 2011)

ALS 10	1p36.22	*TARDBP*	Transactive response DNA binding protein 43 (TDP43)	(Rutherford et al. 2008; Sreedharan et al. 2008)
ALS 11	6q21	*FIG4*	Phosphoinositide 5-phosphatase	(Chow et al. 2009)
ALS 12	10p13	*OPTN*	Optineurin	(Maruyama et al. 2010)
ALS 13	12q24.12	*ATXN2*	Ataxin 2	(Elden et al. 2010)
ALS 14	9p13.3	*VCP*	Valosin containing protein	(Johnson et al. 2010)
ALS 17	3p11.2	*CHMP2B*	Charged multivesicular body protein 2B	(Parkinson et al. 2006)
ALS 18	17p13.2	*PFN1*	Profilin 1	(Wu et al. 2012)
ALS 19	2q34	*ERBB4*	Erb-b2 receptor tyrosine kinase 4	(Takahashi et al. 2013)
ALS 20	12q13	*HNRNPA1*	ROA1/ hnRNPA1 (Heterogeneous nuclear ribonucleoprotein A1)	(Kim et al. 2013a)
ALS 21	5q31.2	*MATR3*	Matrin 3	(Johnson et al. 2014)
ALS 22	2q35	*TUBA4A*	Tubulin α-4A	(Smith et al. 2014)
ALS 23	10q22.3	*ANXA11*	Annexin A11	(Smith et al. 2017)
-	12q24	*DAO*	D-amino acid oxidase	(Mitchell et al. 2010)
-	20q13	*KIAA0693/ CREST/ AA18L1*	Calcium responsive transactivator / synovial sarcoma translocation gene on chr18- like 1	(Teyssou et al. 2013)
ALS- FTD 1	*9p21.2*	*C9ORF72*	Chr9 open reanding frame 72	(Renton et al. 2011; DeJesus-Hernandez et al. 2011)

	ALS- FTD 2	*22q11.23*	*CHCHD10*	Coiled-coil-helix-coiled-coil-helix domain containing protein 10	(Bannwarth et al. 2014)
	ALS- FTD 3	*5q35.3*	*SQSTM1/p62*	Sequestosome 1	(Fecto et al. 2011)
	ALS- FTD 4	*12q14.2*	*TBK1*	TANK-binding kinase 1	(Cirulli et al. 2015)
Autosomal Recessive	ALS 1	21q22.11	*SOD1*	Cu/Zn superoxide dismutase 1	(Al-Chalabi et al. 1998)
	ALS 2	*2q33.1*	*KIAA1563*	Alsin	(Hadano et al. 2001b; Yang et al. 2001)
	ALS 5	*15q15-21.1*	*KIAA1840/ SPG11*	Spatacsin	(Hentati et al. 1998; Orlacchio et al. 2010)
	ALS 6	*16q11.2*	*FUS/TLS*	Fused in sarcoma/ translated in liposarcoma	(Kwiatkowski et al. 2009; Ticozzi et al. 2009)
	ALS 12	*10p13*	*OPTN*	Optineurin	(Goldstein et al. 2016)
	ALS 16	*9p13.3*	*SIGMAR1*	Σ non-opioid intracellular receptor1	(Al-Saif et al. 2011)
	LAHCDA (lateral anterior horn cell disease with arthrogryposis)	*9q34.11*	*GLE1*	GLE1, RNA export mediator	(Kaneb et al. 2015)
X-linked	ALS 15	*Xp11.21*	*UBQLN2*	Ubiquilin 2	(Deng et al. 2011)

Table 5: *Classification of inherited forms of ALS (modified from Mathis et al., 2019).*

ALS 1 – Copper/Zinc Superoxide Dismutase 1 (SOD1)

Mutation of Cu/Zn superoxide dismutase 1 (SOD1) was the first described genetic cause of fALS. In 1991 Siddique and colleagues described the linkage of chromosome 21 (where *SOD1* is located) polymorphisms and ALS (Siddique et al., 1991). Two years later, Rosen and colleagues identified 11 different mutations of this gene (Rosen 1993).

SOD1 encodes a 153 amino acid ubiquitously expressed metalloenzyme. The protein binds copper and zinc to form an extremely stable homodimer. SOD1 dimers are located in the cytosol and intermembrane spaces of mitochondria, providing a vital antioxidant defence mechanism by catalysing the production of oxygen and hydrogen peroxide from the superoxide species produced during cellular respiration (McCord and Fridovich 1969).

SOD1 mutations, detected in 23% of fALS and 2-5% of sALS (Andersen 2006), are characterised by considerable inter and intra-familial variability. E.g. the G37R and L38V variants are associated to an earlier onset and differ from the A4V mutation, which is the most commonly detected and give rise to the most aggressive form of the disease characterised by a rapid disease course; conversely, other variations (e.g. H46R) display a mild phenotype. This evidence suggests that the SOD1 enzyme properties that modulate the timing of symptoms appearance differ from those involved in the rate of disease progression (Cudkowicz et al., 1997). Besides, the penetrance is variable and is strictly dependant on the genetic variant. Most of the *SOD1* mutations are inherited in an AD manner; however, the D90A variant shows both a dominant and recessive pedigree (Andersen 2006).

To date, over 185 disease-associated variations in *SOD1* have been discovered, the majority of which are missense point mutations (Yamashita and Ando 2015). Given that the *SOD1* encodes a 153 amino acid protein, this number is remarkable, suggesting that the modifications are distributed along the gene impacting upon a variety of domains. However, it is not clear whether all the identified variants of *SOD1* are indeed pathogenic (Felbecker et al., 2010).

The multiple mutations identified have resulted in challenges in determining the mechanism through which each alteration affects the disease phenotype. Considering the ~80% of decreased dismutase activity, a loss of enzymatic function was first proposed (Deng et al., 1993; Rosen 1993). However, the subsequent studies showed that the dismutase activity did not correlate with the disease severity, indicating a gain of toxic function of the mutated SOD1 (Reaume et al., 1996). In light of the mutable state of methylation and disulphide bond formation that alter not only the catalytic activity but also its conformational stability, many mutually compatible pathogenic mechanisms to the mutant SOD1 (mSOD1) have been proposed including oxidative stress,

excitotoxicity, protein aggregation, neuroinflammation, apoptosis, mitochondrial dysfunction, axonal transport deregulation and endoplasmic reticulum stress (Kaur et al., 2016). Intriguingly, it has been shown that the mSOD1 can initiate a prion-like seeded aggregation of the wild-type protein (Münch and Bertolotti 2011).

ALS 2 – Alsin

Alsin gene comprises of 33 exons and encodes to a 184KDa protein consisting of 1˙657 amino acids. Predominantly expressed within neurons, Alsin is diffusely distributed, although several dots have been found in cytosol and dendrites (Yamanaka et al., 2003; Hadano et al., 2007). The protein is composed of multiple motifs homologous to guanine-nucleotide exchange factors (GEF). Indeed, it has been shown to function as GEF for Ran, Rho and Rab GTPases (Bischoff 1991). Thanks to this property, Alsin is involved in endosome dynamics, cytoskeleton organisation and neuronal development (Otomo et al. ,2003; Hadano et al., 2007). Interestingly, through its RhoGEF-Pleckstrin domain, Alsin can also specifically binds different variants of the mutant SOD1 (Kanekura et al., 2004).

Mutation in the Alsin gene causes a group of overlapping autosomal recessive neurodegenerative diseases characterised by a long duration/evolution without any bulbar or respiratory signs: infantile onset ascending hereditary spastic paralysis (IAHSP), juvenile primary lateral sclerosis (JPLS) and juvenile ALS (ALS 2). All the pathogenic mutations, that have in common the loss of the C-terminal VSP9 (GEF) domain, led to the production of a truncated protein, suggesting a loss of toxic function mechanism (Hadano et al., 2001a; Yamanaka et al., 2003; Yang et al., 2001). It has been hypothesised that alteration of the long and the short variants of Alsin could lead to ALS, while mutation affecting only the long isoform could cause milder disease as IAHSP and JPLS (Helal et al., 2018). Counterintuitively to the proposed loss of function mechanism, knockout mice for Alsin do not develop any significant motor deficit. However, they are predisposed to oxidative stress, altered vesicles and endosomes trafficking and age-dependent neurological deficit (Cai et al., 2005; Chandran et al., 2008).

ALS 4 – Senataxin

SETX comprises 26 exons and encodes a 302KDa protein of 2˙667 amino acid. Senataxin contains a classical C-terminal 7-motif domain characteristic of the superfamily 1 of DNA/RNA helicases. *SETX* exhibit a strong homology to *RENT1* and *IGHMBP2*, genes involved in the RNA processing. Interestingly *IGHMBP2* mutations are associated with spinal muscular atrophy, a pure LMN disease. Homozygous deletions in *SETX* are linked to ataxia with oculomotor apraxia type 2 (AOA2) and distal hereditary motor neuropathy (dHMN), while heterozygous dominant mutations are associated with ALS 4 (Bennet et al., 2018b).

ALS 4 is characterised by early-onset, a plodding progression and the absence of respiratory and bulbar signs even in advantage stage (Chen et al., 2004; Chance et al., 1998). Considering the different pattern of inheritance of *SETX* mutations in these diseases, ALS4 is probably caused by a gain of toxic function of the mutated Senataxin.

ALS 5 – Spatacsin

Mutation in *SPG11* represents the most common cause of autosomal recessive hereditary spastic paraplegia with thin corpus callosum (Stevanin et al., 2008). However, Orlacchio and colleagues identified 12 frameshift or missense mutations in *SPG11* in 10 unrelated pedigree of ALS (Orlacchio et al., 2010). ALS 5 is characterised by early-onset, a slow progression and distal muscle atrophy associated with pyramidal signs. The protein is composed of 4 transmembrane domains, suggesting its involvement as a receptor or transporter, even if the exact biological function is still missing. Recently, induced pluripotent stem cells (iPS)-derived neuron demonstrated Spatacsin expression within cytoskeleton and that *SPG11* mutation caused axonal dysfunction (Pérez-Brangulí et al., 2014).

ALS 6 – Fused in Sarcoma / Translated in Liposarcoma

FUS encodes a ubiquitously expressed 526 amino acid protein belonging to the FET family of RNA binding protein (RBP). Structurally, presents an N-terminal domain rich in glutamine–glycine–serine–tyrosine (QGSY), three arginine–glycine–glycine (RGG)-rich domains, an RNA recognition

(RRM) and a zinc finger motif, as well as a nuclear export signal (NEL) and nuclear localisation signal (NLS) that enable nucleocytoplasmic shuttling of the protein (Deng et al., 2014).

FUS is involved in several aspects of gene expression (transcription, alternative splicing, transport and translation) (Ratti and Buratti 2016), DNA repair mechanisms (Mastrocola et al., 2013) and also the cellular defence against stress (formation of paraspeckles) (Hennig et al., 2015).

FUS mutations are detected in 4% of fALS and also in 1% of sALS cases. More than 50 autosomal dominant FUS variants have been found in ALS patients. Mostly clustered in the last 18 C-terminal residues (NLS), increasing its retention into the cytosol (Vance et al., 2013), others in the RGG (prion-like) domain and also in the 3'UTR, that increased the propensity of FUS to aggregate (Shang and Huang 2016).

The debate is still ongoing to clarify the loss or gain of function mechanism causing ALS 6. According to the loss of function mechanism, the pathological retainment of the protein within cytosol renders FUS unable to exert its nuclear function. However, FUS knockout models did not show ALS-like phenotype suggesting that loss of FUS is per se not sufficient to cause ALS (Kino et al., 2015). Conversely, as confirmation of the toxic gain of function, mouse overexpressing the wild-type *FUS* developed an aggressive MN degeneration and cytoplasmic FUS accumulation (Mitchell et al., 2013). Discussion is still ongoing concerning whether the toxicity is directly mediated by the FUS aggregates or by the retainment of the insoluble FUS within the cytosol.

ALS 6 patients are characterised by a proximal upper extremities onset, the spreading to the lower without a bulbar region involvement. Neuropathologically, patients exhibit an increased cytoplasmatic FUS staining, with cytoplasmic and neuritic inclusions that do not colocalise with TDP43 (Kwiatkowski et al., 2009).

ALS 8 – Vesicle associated membrane protein B (VAPB)

VAPB comprises 6 exons and encodes a 33KDa protein composed of 243 amino acid. VAPB is a membrane protein localised in plasma and intracellular vesicle membranes and can associate with microtubules. As homodimer (VAMPB) or heterodimer (associated with VAMPA), the complex interacts with synaptobrevin 1 and 2 (VAMP1 and VAMP2) regulating the vesicular trafficking (Weir

et al., 1998). Furthermore, like type 2 integral endoplasmic reticulum (ER) membrane protein, is involved in the unfolded protein response and in regulating the ER-mitochondria interaction (Lev et al., 2008).

Linkage analysis of a large Brazilian family put VAPB forward as a causative gene of ALS. In particular, the P56S variant has been identified in multiple Brazilian pedigrees suggesting a joint founder (Nishimura et al., 2005). The P56S mutant VAPB is characterised by an impaired unfolded protein response, altered calcium buffering and disrupted anterograde axonal transport of mitochondria (Mórotz et al., 2012a). Several mutations have been discovered during the last years, though not all segregated with the disease (Kabashi et al., 2013; van Blitterswijk et al., 2012).

ALS 8 phenotype is characterised by a slow progression of the disease, LMN symptoms (tremor, cramps, fasciculations) without the involvement of UMN (Nishimura et al., 2004).

ALS 9 – Angiogenin (ANG)

Angiogenin, a.k.a ribonuclease 5, is a small 123 amino acid protein. Upon the binding to the cognate surface receptor, angiogenin is internalised and translocated to the nucleus where stimulates several biological pathways, including tRNA (transfer RNA) transcription, ribosome biogenesis, cell proliferation, etc. (Moroianu and Riordan 1994). Recent evidence reported a pivotal role of angiogenin in the assembly of stress granules. Interestingly, the G-quadruplets structures formed by the G_4C_2 *C9ORF72* expansion inhibits this mechanism, thereby establishing a connection between these two genes (Ivanov et al., 2014).

ANG mutations, present in 2% of fALS and 0.8% of sALS patients, lead to inhibition of angiogenin secretion and impairment of its numerous functions finally causing MN degeneration (Greenway et al., 2006).

ALS 10 – TAR-DNA binding protein 43 (TDP43)

TARDBP encodes several protein isoforms, among which TDP43 is the most prevalent.

TDP43 is a 414 amino acid heterogeneous nuclear ribonucleoprotein (hnRNP) containing a nuclear localisation (NLS) and nuclear export signal, which allow shuttling of the protein between the nucleus and the cytosol. TDP43 is composed of different domains, two RNA recognition motifs,

involved in nucleic acid binding, and a C-terminal glycine-rich domain (prion-like domain) which is essential for the protein-protein interaction (Ayala et al., 2008; Baralle et al., 2013).

TDP43 is a regulator of gene expression; therefore it has been shown to play a pivotal role in RNA metabolism (transcription, splicing, transport, etc.) (Scotter et al., 2015; Ratti and Buratti 2016). Recently, it has been found as a component of stress granules, suggesting its involvement also in cell protection from damage (Aulas and Velde 2015). However, the real biological function of TDP43 is still unknown.

To date, at least 48 variants in *TARDBP* have been associated with ALS, the majority of which are missense mutations located in the C-terminal of the transcript (Lattante et al., 2013).

TDP43 is the main component of the characteristic ubiquitinated inclusions observed in patients with ALS (97%) and FTD. This evidence establishes TDP43 as the prominent protein signature of ALS, not just in the TDP43 mutation carrier patients (Neumann et al., 2006; Schipper et al., 2016). The aggregated forms of TDP43 are characterised by abnormal phosphorylation (and/or post-translational modifications), truncation and mislocalisation in the cytosol. Neurodegeneration is probably due to one or all the properties acquired by the mutant TDP43: gain of toxic function, loss of function or aberrant function (Alsultan et al., 2016).

ALS 11 – Phosphoinositide 5-phosphatase (FIG4)

FIG4, also known as SAC3, regulates PI(3,5)P2 (phosphatidylinositol 3,5-bisphosphate) levels and thereby controls retrograde trafficking of endosomal vesicles to Golgi. The mutant proteins showed loss of phosphatase activity, mislocalisation, and inability to bind to the PI(3,5)P2 complex.

Mutations in *FIG4* were originally identified in Charcot-Marie-Tooth patients; however, screening of ALS patients identified nine variants that possibly led to a FIG4 mislocalisation (Chow et al., 2009). Phenotypically, ALS 11 showed a longer disease duration with UMN predominance (Osmanovic et al., 2017).

ALS 12 – Optineurin (OPTN)

OPTN gene encodes the coiled-coil containing protein Optineurin of 67KDa. Optineurin is involved in the autophagosome transport, through the interaction with ubiquitin and ubiquilin2, in the Golgi organisation and the regulation of NF-κB signalling (Bansal et al., 2015).

OPTN mutation causes an exaggerated activation of NF-κB, altering the neuronal function and accelerating the body inclusions formation (Maruyama et al., 2010).

OPTN variants (exonic deletion and nonsense mutation) were initially identified in Japanese consanguineous. Subsequent screening has identified additional heterozygote mutations in fALS and sALS cases (Goldstein et al., 2016).

ALS 13 – Ataxin 2 (ATX2N)

ATXN2 contains 9 exons, two of which are protein-coding. The CAG repeat in the coding sequence is prone to error in DNA replication, and its length can vary widely between individuals. More than 36 CAG repeats are associated with spinocerebellar ataxia 2. However, a recent meta-analysis has identified 25-28 repeats as protective, whilst a significant risk to develop ALS was associated with 31-33 CAG repeats (Elden et al., 2010; Neuenschwander et al., 2014).

ATXN2 is an RNA binding protein localised within the endoplasmic reticulum, Golgi and stress granules ad it is involved in RNA processing.

ALS 13 is characterised by spinal onset and shorter survival (Borghero et al., 2015).

ALS 14 – Valosin containing protein (VCP)

VCP is an AAA+ ATPase protein involved in various cell activities, including the mediation in the degradation of ubiquitinated protein by the proteasome and the targeting of substrates to the autophagosome. Therefore, the discovery of VCP mutations highlighted the involvement of ubiquitination/protein degradation defects in ALS pathogenesis (Meyer and Weihl 2014).

Initially, an exome sequencing analysis put *VCP* variants forward as causative in ALS. Subsequent studies identified further four *VCP* variants providing the evidence of a clear association with fALS (Johnson et al., 2010). *VCP* mutations have also been found in a rare form of Paget Disease (IBMPFD, Inclusion Body Myopathy with Paget disease of bone and Frontotemporal Dementia), which is

characterised by mitochondrial uncoupling and a reduced ATP production, common features of ALS 16 (Kimonis et al., 2008).

ALS 15 – Ubiquilin 2 (UBQLN2)

UBQLN2 encodes for Ubiquilin 2 protein that contains an N-terminal ubiquitin-like domain and a C-terminal ubiquitin-associated domain. Ubiquilin lays an essential role in the regulation of different protein degradation mechanisms and pathways including ubiquitin-proteasome system (UPS), autophagy and the endoplasmic reticulum-associated protein degradation (ERAD) pathway (Kleijnen et al., 2000; Xia et al., 2014).

Mutations in *UBQLN2*, mostly within the PXX repeat region, have been shown to disrupt the protein degradation pathway, causing the mislocalisation of OPTN from endosomal vesicles and also impairing the RNA metabolism, through the loss of hnRNP (heterogeneous nuclear ribonucleoproteins) binding (Gilpin et al., 2015). These observations highlight how the protein turnover and RNA metabolism impairment fulfil a pivotal role in ALS pathogenesis.

ALS 16 – Sigma-1 Receptor (SIGMAR1)

SIGMAR1 encodes for the sigma-1 receptor, which is involved in endoplasmic reticulum stress, calcium transport within mitochondria and chaperone activity. Mutation of *SIGMAR1* causes the formation of cytoplasmic aggregations, reduction in ATP production and subsequent decrease in proteasome activity (Fukunaga et al., 2015).

Initially, 3'-UTR variants were observed in FTD-ALS or pure FTD families, suggesting the alteration of the RNA metabolism as the leading mechanism (Luty et al., 2010). Subsequent studies identified a missense mutation segregating in a large family with autosomal dominant juvenile ALS (Al-Saif et al., 2011). However, the contribution of *SIGMAR1* mutations in ALS needs further investigations.

ALS 17 – Charged multivesicular body protein 2B (CHMP2B)

CHMP2B encodes a component of the heteromeric ESCRT-III complex (Endosomal Sorting Complex Required for Transport III) that functions in the recycling or degradation of cell surface receptors. CHMP2B is found as a monomer in the cytosol or as an oligomer in ESCRT-III complexes on endosomal membranes.

CHMP2B variants have been identified in both fALS and sALS patients, the majority of which showed an LMN phenotype (Cox et al., 2010).

ALS 18 – Profilin 1 (PFN1)

PFN1 encodes for an actin-binding protein, that plays an essential role in actin dynamics by regulating its polymerisation in response to extracellular stimuli. Moreover, it has been shown a profilin 1 co-localisation within stress granules, suggesting a role in cell defence from damage (Figley et al., 2014).

Several *PFN1* variants have been found in fALS and sALS patients, with the pE117G mutation identified as a risk factor (Wu et al., 2012; Fratta et al., 2014).

PFN1 mutations destabilise the protein function, although the loss of function/gain of toxic function mechanism is yet to be clarified.

ALS 19 – Erb-b2 receptor tyrosine kinase 4 (ERBB4)

ERBB4 is one of the four members in the EGFR subfamily of receptor tyrosine kinases. It is specifically bound by neuregulins (NRG3 and NRG4) resulting in the autophosphorylation of the C-terminal.

ERBB4 was found to localise to interneurons C-boutons which synapse with spinal MNs. Interestingly, C-boutons are not present in oculomotor neurons, which are spared from the disease (Gallart-Palau et al., 2014).

ERBB4 mutations, found in both fALS and sALS, decrease the protein activation through the inhibition of C-terminal autophosphorylation (Takahashi et al., 2013).

ALS 20 – Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1)

HNRNPA1 encodes for a member of a family of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs). hnRNPA1 is one of the most abundant core proteins of hnRNP complexes and plays a crucial role in the regulation of RNA alternative splicing, but also cell apoptosis. Interestingly, hnRNP1 interacts with TDP43 and ubiquilin 2 (Gilpin et al., 2015).

Although histopathological analysis showed intense nuclear staining of hnRNPA1 within MN perikaryon, that correlates with nuclear loss of TDP43, a wide screening study of fALS and sALS patients suggested that this is an infrequent cause of the disease (Alsultan et al., 2016).

ALS 21 – Matrin 3 (MATR3)

MATR3 encodes for a protein with RNA and DNA binding domains that appears to be involved in regulating the gene expression (Coelho et al., 2015).

Exome sequencing studies have identified four *MATR3* mutations in fALS and eleven in sALS pedigree, that account for less than 1% of ALS (Johnson et al., 2014; Marangi et al., 2017)

It has been recently reported that matrin 3 forms complex with other ALS-associated RNA binding proteins (RBP) such as FUS and TDP43 (Yamaguchi and Takanashi 2016; Johnson et al., 2014). Interestingly, the p.S85C variant increases the interaction whit the RBPs while other mutations (pF115C, p.P154S and p.T622A) do not (Johnson et al., 2014). Nevertheless, by contrast to TDP43 and FUS, the subcellular localisation of the mutant matrin 3 is unaffected.

Arguably, the different variants hitherto discovered and their intrinsic property of interaction with other proteins might be responsible for the heterogeneous phenotype of ALS 21 (Chia et al., 2018).

ALS 22 – Tubulin-α4A (TUBA4A)

TUBA4A encodes tubulin-α4A, a protein involved in cytoskeletal structural dynamics.

Exome sequencing analysis discovered four missense and two nonsense mutations, four of which deleterious, that account about 1% of fALS and 0.4% of sALS cases (Smith et al., 2014).

In vitro studies showed that mutant tubulin-α4A is inefficient at forming αtubulin-βtubulin dimers, which are poorly incorporated into microtubules thus reducing the structural stability of cytoskeleton, in the interaction with the axonal transport proteins dynein and kinesin, and promotes the depositions of ubiquitinated cytoplasmic inclusion (White and Sreedharan 2016; Smith et al. 2014). These pieces of evidence highlight the crucial role of cytoskeletal and axonal defects in ALS pathogenesis.

Scant information is available concerning the clinical phenotype of ALS 22. Although patients often develop ALS typical features, some display common symptoms of FTD (Smith et al., 2014).

ALS 23 – Annexin 11 (ANXA11)

ANXA11 encodes a 56KDa protein member of the annexin family, a group of calcium-dependent phospholipid-binding proteins, which are involved in vesicular trafficking between Golgi and ER. Annexins have unique N-terminal domains and conserved C-terminal domains, which contain calcium-dependent phospholipid-binding sites.

Physiologically, annexin 11 localised in cytoplasmic vesicle-like structures and foci that are widely distributed throughout the somata, dendrites and axons. *In vitro* studies showed that the mutant annexin 11 tends to aggregate, due to the loss of its binding propensity for calcyclin (a protein involved in proteostasis), and loses association with the vesicle-like structures. Moreover, as a prion-like mechanism, the mutant annexin 11 sequesters the wild-type protein inhibiting its biological activity (Smith et al., 2017).

Mutations in ANXA11 were observed in about 1% fALS and 1.7% sALS patients (Nguyen et al., 2018).

ALS-FTD 1 – Chromosome 9 open reading frame 72 (C9ORF72)

Discovered in 2011, the hexanucleotide repeat expansion (G_4C_2) in the non-coding region of the intron 1 of *C9ORF72* represents the most common inherited cause of ALS in Europe, but not in Asia (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Majounie et al., 2012).

While healthy controls commonly have <10 G_4C_2 repeats, ALS patients carrier 400-2˙000 repeats. The repeat expansion has been found in 37.6% fALS and 6.3% sALS patients, as well as in up to 25.1% of FTD cases (Majounie et al., 2012). Accordingly, the clinical phenotype of this ALS subtype is strongly associated with FTD. Moreover, it has been reported that ALS-FTD 1 patients exhibit a higher incidence of bulbar onset and earlier symptoms manifestation compared to non-C9ORF72 ALS subjects (Cooper-Knock et al., 2015).

Recent studies have shown that C9ORF72 has several biological functions. Structural analysis revealed a similarity to GDP/GTP exchange factors that regulate Rab proteins and thus the vesicular trafficking (Levine et al., 2013), while colocalisation studies candidates C9ORF72 involvement in autophagy and endosomal trafficking (Farg et al., 2014).

Several hypotheses have been proposed to explain how the intronic hexanucleotide expansion may cause neurodegeneration:

- ✓ Inhibition of endosomal trafficking and perturbation of endocytosis (leading to autophagy) caused by a protein loss of function. However, knock out mice do not develop motor neurons degeneration, suggesting that the loss of C9ORF72 function alone is not the primary cause of ALS-FTD 1 (Koppers et al., 2015).
- ✓ Formation of RNA foci dependent from the expansion length, in which RNA binding proteins are sequestered resulting in the disabling of the RNA processing machinery (Lee et al., 2013; Todd and Petrucelli 2016).
- ✓ Production of five different dipeptide repeat (DPR) proteins through RAN (non-AUG) translation, which showed a strong propensity to aggregation. Of all (polyGA, polyGP, polyGR, polyPA and polyPR), polyGA exhibits the most toxic power through the activation of programmed cell death and the dose-dependent cleavage of TDP43, another ALS-associated protein (Lee et al., 2017).
- ✓ Disruption of the nucleocytoplasmic shuttling at the level of the short isoform (C9-S), generally localised at the nuclear membrane. Moreover, it has been demonstrated a correlation between the loss of C9-S and the TDP43 mislocalisation (Mihevc et al., 2017).
- ✓ Increased vulnerability to calcium-permeable AMPA receptor-mediated excitotoxicity (Selvaraj et al., 2018).

Finally, in light of the haploinsufficiency characteristic of ALS-FDT 1 patients, the neurodegenerative phenomenon might be driven by al least two mechanisms: excitotoxicity and impaired clearance of neurotoxic DPR (Shi et al., 2018). Therefore, in ALS-FDT 1, the degenerative events are driven by both a loss and a gain of toxic function.

ALS-FTD 2 – Coiled-coil helix coiled-coil helix domain-containing protein 10 (CHCHD10)

CHCHD10 encodes 14KDa mitochondrial protein which, together with mitofil, CHCHD3 and CHCHD6, forms the multiprotein complex MICOS (mitochondrial contact site and cristae organizing system), which is pivotal in the formation and maintenance of cristae structure.

Twentyone *CHCHD10* variants clustered in the exon 2, which encodes an internal hydrophobic helical segment important for mitochondrial membrane binding, have been found in a broad range of neurodegenerative disorders including ALS and FTD (Bannwarth et al., 2014; Zhang et al., 2015b). Analyses performed in fibroblast from subjects with *CHCHD10* mutation showed structurally abnormal mitochondria and defects in the respiratory chain and mitochondrial genome stability (Bannwarth et al., 2014). These alterations are, as the mitochondrial abnormalities, observed in mutant TDP43 patients. Physiologically CHCHD10 interacts with TDP43 promoting its nuclear retention; however, the mutant CHCHD10 lose this ability augmenting the TDP43 accumulation within the cytoplasm (Woo et al., 2017).

Nevertheless, *CHCHD10* mutations appear to be a relatively rare cause of ALS (1%) but might be more frequent among FTD patients (10%) (White and Sreedharan 2016).

ALS-FTD 3 – Sequestosome 1 (SQSTM1/p62)

SQSTM1 encodes a ubiquitin-binding protein that plays a role in protein degradation via autophagy and proteasome. The protein functions as an adaptor, in concert with TNF-receptor associated factor 6, to mediate the activation of NF-κB in response to upstream signals.

Several *SQSTM1* variants that led to a loss of protein function have found in fALS and sALS patients but also Paget disease (Teyssou et al., 2013; Fecto et al., 2011).

ALS-FTD 4 – TANK-binding kinase 1 (TBK1)

TBK1 encodes for a homodimeric multidomain protein containing a kinase domain, a ubiquitin-like domain and two coiled-coil domains.

TBK1 interacts with several proteins and regulates numerous critical cellular processes involved in ALS including neuroinflammation, ubiquitin-proteasome systems and autophagy pathways engaging other genes ALS-associated (i.e. *OPTN*, *SQSTM1*, *VCP*, and *UBQLN2*) (Oakes et al., 2017).

Most mutations localised in the coiled-coil and kinase domains leading to the loss of function of TBK1 and resulting in the alteration of the downstream regulatory pathways.

Patients with mutant *TBK1* exhibit haploinsufficiency of the protein (Freischmidt et al., 2015), and are characterised by bulbar onset, cognitive impairment. Moreover, TBK1 carrier showed TDP43

inclusions in the brain and spinal cord, thus listing TBK1-ALS as another "TDP43 proteinopathy" (White and Sreedharan 2016).

TBK1 variants account 1.3% of fALS, 1% of sALS and 4% of ALS-FTD patients (Nguyen et al., 2018). Moreover, ALS patients that exhibit FTD signs and *TBK1* mutation are negative for *C9ORF72* mutation, candidating *TBK1* as an ALS causative gene (Nguyen et al., 2018).

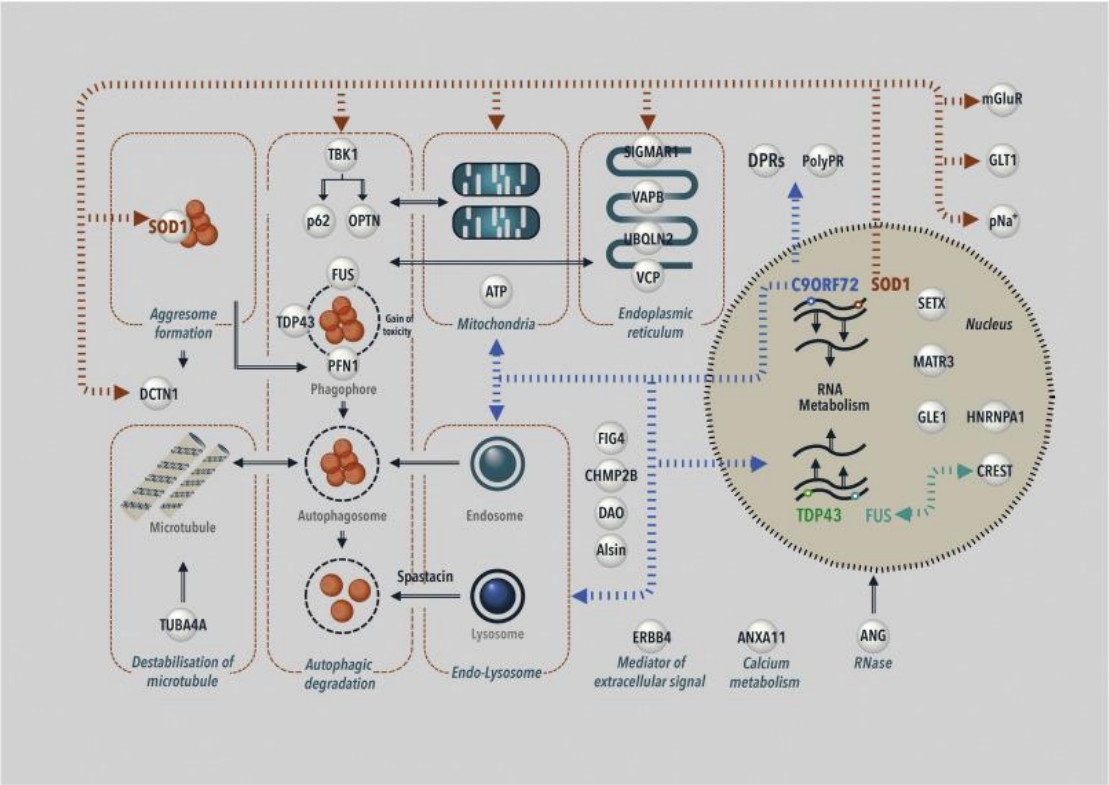

Figure 1: *Localisation and role of the targets (proteins) of the main causative genes of fALS* (Mathis et al. 2019).

Altogether the genetic determinants of fALS can also explain more than 20% of sALS.

These data are represented in Table 6.

Denomination	*Gene*	*fALS*	*sALS*
ALS 1	*SOD1*	20%	2-5%
ALS 2	*ALSIN*	rare	no association
ALS 3	Unknown (18q21 locus)	rare	unknown
ALS 4	*SENATAXIN*	rare	rare
ALS 5	*SPATACSIN*	rare	unknown
ALS 6	*FUS/TLS*	4%	1%
ALS 7	Unknown (20p13 locus)	rare	unknown
ALS 8	*VAPB*	rare	unknown

ALS 9	*ANG*	2%	rare
ALS 10	*TARDBP*	3-6%	1.5%
ALS 11	*FIG4*	rare	rare
ALS 12	*OPTN*	1-4%	rare
ALS 13	*ATXN2*	rare	rare
ALS 14	*VCP*	1-2%	rare
ALS 15	*UBQLN*	1%	rare
ALS 16	*SIGMAR1*	unknown	no association
ALS 17	*CHMP2B*	1%	rare
ALS 18	*PFN1*	2-3%	rare
ALS 19	*ERBB4*	unknown	unknown
ALS 20	*HNRNPA1*	rare	rare
ALS 21	*MATR3*	1%	rare
ALS 22	*TUBA4A*	1%	rare
ALS 23	*ANXA11*	1%	2%
ALS FTD 1	*C9ORF72*	38%	7-10%
ALS FTD 2	*CHCHD10*	1%	unknown
ALS FTD 3	*SQSTM1*	1%	rare
ALS FTD 4	*TBK1*	2-3%	1-2%

Table 6: *Common genetic determinants of fALS and sALS.*

Sporadic ALS (sALS)

The aetiology of ALS remains mostly unknown. However, the epidemiological data hitherto collected indicate that genetic factors highly contribute to its pathogenesis. Indeed, genetic mutations initially observed in fALS pedigree have also been identified in (apparently) sALS patients, as reported in Table 6.

Nevertheless, the identification of gene variants in sALS cases has met with limited success so far. Studies performed to link particular genetic variants to sALS represent a small number of cases, reflecting the intricate pattern of inheritance, the high heterogeneity in the clinical manifestation and the presence of environmental risk factors in the disease. However, these studies highlighted the presence of “susceptibility genes” that, once mutated, might increase the risk to develop the

disease probably due to the ability of the encoded mutant protein to dysregulate the interaction with other ALS-associated pathways.

The primary discovered gene are listed in the table below.

Gene	Protein	Biological function	Reference
DCTN1	Dynactin	ER-Golgi transport, lysosome and endosome trafficking, chromosome movement and axonogenesis	(Münch et al., 2004)
NEFH, NEFM, NEFL	Neurofilament subunits	Axons support	(Figlewicz et al. 1994)
PRPH	Peripherin	Neurofilament assembly	(Gros-Louis et al. 2004)
VEGF	Vascular endothelial growth factor	Angiogenesis, vascular permeability, neuronal growth and repair	(Lambrechts et al. 2003)
SMN	Survival motor neuron	RNA processing	(Veldink et al. 2005)
CTNF	Ciliary neurotrophic factor	Neurotransmitter synthesis, neurite outgrowth and neuronal trophic factor	(Giess et al. 2002)
APOE	Apolipoprotein E	Lipoprotein metabolism	(Moulard et al. 1996)
APEX	DNA repair enzyme apurinic/apyrimidinic endonuclease	Gene expression regulation, cellular response to stress	(Kisby et al. 1997)
HFE	Homeostatic iron regulator	Cellular response to oxidative stress	(Wang et al. 2004)

Table 7: *Most common sALS "susceptibility genes".*

In conclusion, increasing the knowledge about the genetic profile that represents a "risk" or "protective" factor in ALS will change the way clinical trials are done and how therapy is prescribed to patients. The complete characterisation of the genetic profile of patients will allow the correct stratification of cohorts and thus increase the success of clinical trials. Finally, raising awareness about the genetic profile of patients will be a breakthrough to integrate the genetic screening for known variants/mutations to diagnosis, treatment and prevention of ALS.

1.1.6 THERAPY

ALS is a poor prognosis disease without an effective treatment, this due to the several pathogenetic mechanisms involved and since the main feature of ALS in the high phenotypic variability (Bendotti et al., 2020).

Recently, two treatments have been recognised and classified as "disease-modifying". The longest available, FDA (Food and Drug Administration) approved in 1995, is the anti-glutamatergic agent Riluzole, which prolongs the lifespan of patients of about 3-6 months without affecting the quality of life (Miller et al., 2001).

The other disease-modifying treatment, recently approved by FDA, but not by EMA (European Medicines Agency), is Edaravone (Radicut®), an antioxidant agent able to eliminate lipid peroxides and hydroxyl radicals (Bhandari et al., 2018).

A massive number of therapies have been/are being studied on different stages of preclinical and clinical practice. They are categorised based on the pathophysiological mechanism and listed below.

Anti-apoptotic

This group of treatment focuses on mechanisms leading to motor neuron death (e.g. mitochondrial impairment, abnormal calcium handling, etc..). In this class are included Dexpramipexole (Cudkowicz et al., 2013) with failed outcomes, and more recently Ursodeoxycholic and Tauroursodeoxycholic acids, both with moderate positive results (Elia et al. 2016; Min et al. 2012).

Anti-inflammatory

Mounting evidence shows that neuroinflammation process associated with reactive glial and infiltrating immune cells plays a pivotal role in ALS determination and progression. Forasmuch as inflammation has emerged as a critical mechanism in driving the disease, several immunomodulatory therapies have been hitherto tested in ALS patients (Wosiski-Kuhn et al., 2019). Mentioned among treatments targeting the innate immune system: Celecoxib, cyclooxygenase 2 (COX2) inhibitor that protects from excitotoxicity blocking the prostaglandins synthesis (Cudkowicz et al., 2006); Minocycline, antibiotic that reduces microglial activation and polarises the

macrophages toward an anti-inflammatory phenotype (Gordon et al., 2007); Glatiramer Acetate and RN60, compounds able to tip the balance toward the alternative (Th2, M2) inflammatory response (Gordon et al., 2006; Paganoni et al., 2019).

Conversely, some treatments have been tested to attempt in modulating the adaptive immune response, chiefly T lymphocytes. An example is the total lymphoid irradiation (TLI), which, through the selective target of lymphoid organs, abolish the circulating lymphocytes. However, this treatment did not obtain the expected outcome in ameliorating the disease course of patients but rather increased the number of circulating $CD8^+$ cytotoxic T cells (Drachman et al., 1994). Another drug able to inhibit the egress of lymphocytes from lymph nodes and recently tested in ALS is Fingolimod, which, differently from TLI, do non affect the level of circulating T regulatory cells (Berry et al., 2017).

Efforts were also made to target the link between the innate and adaptive immune system: the cytokines. Examples of this class of drugs are Anakinra, a selective interleukin 1 receptor (IL1-R) antagonist (Maier et al., 2015), and Tocilizumab, a monoclonal antibody against IL-6 (Mizwicki et al., 2012).

Anti-excitotoxicity

In ALS, excitotoxicity is derived from an excessive glutamate release combined with alterations in post-synaptic glutamatergic receptors and transporters. Directly involved in the inhibition of the excitotoxic phenomenon in ALS are, as mentioned before, Riluzole, Ceftriaxone (Cudkowicz et al., 2014), Memantine (de Carvalho et al., 2010) and Methylcobalamin (Kaji et al., 2019).

Antioxidant

Oxidative stress represents one of the most prominent factors playing a pivotal role in ALS pathogenesis. Several antioxidant compounds have been tested, including the above mentioned Edaravone and Pramipexole (Pattee et al., 2003).

Anti-aggregation

The protein aggregation and deposition (in particular TDP43 and mutantSOD1) is a hallmark of ALS; indeed, it is considered a proteinopathy. Preclinical evidence candidates as anti-aggregant factors:

Arimoclomol, an amplifier of heat shock protein-mediated response (Lanka et al., 2009), MIF (macrophage migration inhibitory factor), a compound able to inhibit the toxic misfolded SOD1 aggregates (Shvil et al., 2018) and an acridine derivate [4,5-bis{(N-carboxy methyl imidazolium)methyl}acridine] that seems to antagonised the TDP43 aggregation (Afroz et al., 2017).

Neuroprotection (neurotrophic factors)

Besides inhibiting the numerous pathological mechanisms underlying neurodegeneration in ALS, efforts have been made to identify therapies that could stimulate the repair of damage MN or promote the growth of new ones. Recently have been proposed as promising compounds: 7,8-DHF (7,8-dihydroxyflavone), an agonist of tyrosine kinase receptor B (TkrB) that mimic the effects of BDNF (brain-derived neurotrophic factor) (Korkmaz et al., 2014) and GPNMB (glycoprotein non-metastatic protein B), which seems to reduce the TDP43-mediated stress (Tanaka et al., 2012).

Muscle strength

Although ALS is an MND, the first symptoms appear at the muscular level (Moloney et al., 2014). Moreover, the primary cause of death in ALS patients is respiratory failure due to the progressive weakening of diaphragm. Therefore, maintain and/or increase the muscle strength and functionality might significantly ameliorate the disease progression.

For this purpose have been proposed: the soluble form of activin IIB receptor, an inhibitor of negative regulators of muscle growth (e.g. GDF8 myostatin) (Morrison et al., 2009) and Tirasemtiv, a troponin activator (Shefner et al., 2019).

Cell-based therapy

Nowadays, stem cells approaches are primarily designed to increase neuroprotection (paracrine effect) rather than to replace degenerated neurons.

Mesenchymal stromal cells (MSC) are primarily being used as an autologous stem cell therapy due to their ability to secrete neurotrophic factors and modulate the immune system, as demonstrated in several preclinical and clinical studies (Bonafede and Mariotti 2017).

Another stem cell strategy implies the use of glial-restricted precursors or neural progenitor stem cells (Lepore et al., 2011; Edwards 2016). However, the demonstration that only a small amount of

the injected cells can engraft and differentiate within the injury site suggested that their beneficial effect was indirectly and thus mediated by the several factors released. Subsequent studies demonstrated that stem cells produce a broad spectrum of extracellular vesicles (EVs) containing an enormous amount of factors (cytokines, growth factors, nucleic acids, etc.). This evidence indicated that stem cells could exert their beneficial effect through the EVs secretions, which promote the wound healing releasing their content within the damaged area (Baglio et al., 2012). Thus, EVs (i.e. microvesicles and exosomes) could be used as a novel therapeutic tool, avoiding the ethical and immunogenic risks of stem cells (Bonafede and Mariotti 2017).

Gene therapy

The progress of medicine brings alternative and innovative approaches for the treatment of so far incurable neurodegenerative diseases, including ALS.

Targeting ALS-associated genes, genetic modifiers or related disease molecules have shown promising results (Cappella et al., 2019). Indeed, it has been shown that antisense oligonucleotides (ASOs) against SOD1 were able to eliminate the mutant protein without adverse effects (Miller et al., 2013). One disadvantage of ASOs is the need for repetitive infusions or the identification of the correct dose. The deliver of ASO or short hairpin RNA to knockdown mutant SOD1 through a viral vector (e.g. AAV9, adeno-associated virus serotype 9) circumvents this issue (Foust et al., 2013; Iannitti et al., 2018). Another approach currently under consideration is the delivery of an AAV9 expressing a single-chain antibody against misfolded SOD1, which demonstrated its efficacy postponing the disease onset and extending survival in ALS mice (Maier et al., 2018).

Although these studies were focused on SOD1 ALS-related gene, these approaches can potentially be applied to others known ALS-causing gene or related disease molecules, thus increasing their relevancy also for sALS cases. Indeed, it has been recently reported an amelioration of clinical phenotype of ALS mice following the administration of ASOs, an interfering RNA (RNAi) or using a CRISPR/Cas9 technique to targeting the G4C2 expansion of *C9ORF72* gene (Jiang et al., 2016; Martier et al., 2019; Pickles and Petrucelli 2018).

Moreover, for sALS patients a more general neuroprotective approach was winnowed consisting in the delivery of growth factors such as VEGF (vascular endothelial growth factor), IGF1 (insulin-like growth factor 1), GCSF/CSF3 (granulocyte-colony stimulating factor/colony-stimulating factor 3) (Dodge et al. 2010; Henriques et al. 2011).

Despite the efforts done in basic research and clinical trials, ALS remains a poor prognosis disease, and only two disease-modifying therapies are available to date.

This failure could have been caused by the use of animal models of ALS. As far as representing a useful tool, small rodents do not mimic the heterogeneity of the human disease faithfully and, although their genome is closely related to our, animal models do not exhibit precisely the same modifications of humans. To overcome this issues, iPS cells (induced pluripotent stem cells) derived from ALS patients or control subjects, represent a promising *in vitro* platform for discovering unique "human neuron phenotypes" that may reflect the individual disease and, thus, testing newly-discovered therapeutic approach (Engle et al., 2018; Morgan et al., 2018).

Moreover, ALS is considered a multifactorial and multisystemic disease in which MN death represents the final event. Therefore, the purpose of drug combinations would appear to be a logical approach, even if this strategy remains largely unexplored both in preclinical and clinical studies.

1.2 PATHOGENETIC MECHANISMS

Despite decades of basic research, causative mechanisms in ALS remain elusive. Studies performed in autoptic human samples and animal models suggest that it is likely that multiple pathogenetic mechanisms, rather than a single trigger event, actively participate in the determination and progression of ALS.

Moreover, as discussed above, it is known only the 5% of causes of ALS (fALS) while the remaining 95% (sALS) are still undisclosed. The genetic but also the phenotypic variability represents an enormous confounding factor in uncovering and drawing conclusion regarding the pathogenetic mechanisms underlying ALS. Nevertheless, since the clinical and pathological profile of fALS and

sALS patients are indistinguishable, it can be predicted that the evidence obtained from the studies performed on animal models of ALS (i.e. fALS) may be acceptable also to sALS patients.

However, more clarity is needed concerning the timing and extent to which each of the pathogenetic mechanisms listed below is involved and actively contribute to ALS development and progression.

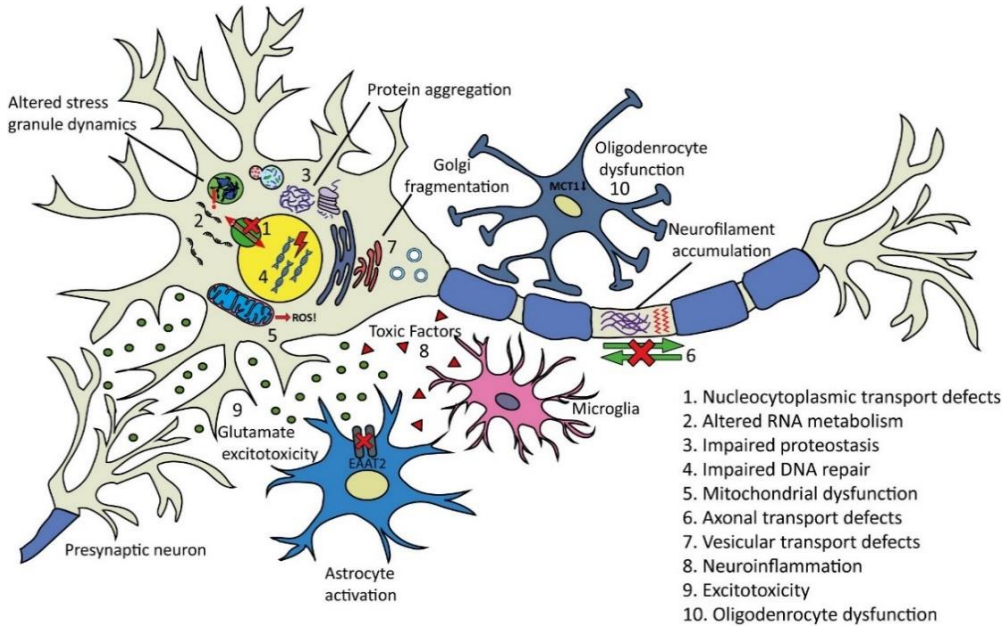

Figure 2: *Proposed pathogenic mechanisms and pathology in ALS* (Mejzini et al. 2019).

1.2.1 MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS

The term reactive oxygen species (ROS) comprehends hydroxyl radicals (OH^-), peroxynitrite ($ONOO^-$), superoxide radical anion (O_2^-), nitric oxide (NO) and hydrogen peroxide (H_2O_2). Aerobic metabolism (mitochondrial respiratory chain) is the primary source of free radicals. Still, they are also produced by cytochrome P450 enzymes in the ER or by immune cells as a second messenger (Knight 2000). Therefore, at moderate levels, ROS have beneficial effects and are involved in various physiological functions, such as immune functions (i.e. defence against pathogens), cellular signalling pathways, mitogenic response and redox regulation (Valko et al., 2007).

Oxidative stress may occur, on one side, when an imbalance between antioxidant capacity and the rate of ROS production occurs or, on the other side, when the cell antioxidant capacity is impaired due to a deficiency of enzymatic and non-enzymatic antioxidants activity. When antioxidants do

not neutralise ROS, the latter can seriously damage the integrity of cell homeostasis affecting the stability of various biomolecules (Thanan et al., 2014).

Mounting evidence showed the involvement of oxidative stress in ALS (Barber et al., 2006). Indeed, oxidative stress markers have been observed in both patients (Barber and Shaw 2010; Wang et al., 2019) and disease models (Cacabelos et al., 2016). However, it is not known yet if oxidative stress represents a causative event in ALS degenerative cascade or it is merely a consequence of other toxic insults.

Possible trigger events could be ageing. Indeed, ALS is an adult-onset disease and several pieces of evidence reported an increased ROS production during ageing (Liguori et al., 2018). Although ageing is unlikely to be the cause of disease, in some patients it could represent a risk factor as the origin of an excessive cellular response to toxic stimuli that finally leads to MN degeneration (Barber and Shaw 2010).

Mitochondrial are at the same time the primary source and the main target of ROS, establishing a vicious circle (Lin and Beal 2006). The overload of reactive species increases the mutations in mitochondrial DNA (mtDNA) altering the production of components of the respiratory complexes, this leads to an impairment of mitochondrial detoxifying function finally resulting in further ROS production (Genova et al. 2004).

Therefore, researchers focused on the comprehension of the mitochondria-related pathogenic mechanisms in ALS. These studies showed that the mitochondrial functions can be dysregulated by the aggregating products of ALS-related genes and/or by the aberrant RNA processing and that products cannot be eliminated by cell due to the impaired autophagic mechanism (Carrì et al., 2017; Smith et al., 2019).

SOD1 has been the first ALS-related gene discovered and encodes for the main anti-oxidative enzyme of cells. This evidence confirms the involvement of SOD1 protein in ALS pathogenesis, suggesting its participation also in the oxidative stress phenomenon. Interestingly, preclinical studies demonstrated that ALS pathogenesis might involve not only the decrease/loss of antioxidant activity of mutant SOD1 but also the acquirement of a toxic gain of function caused by

an altered geometry of the active site that allows the entry of reducing substrates (Rakhit and Chakrabartty 2006). This alteration results in increased ROS production and a modified interaction with mitochondria and other proteins (Cozzolino et al., 2009). Besides, it has been reported that mutant SOD1 alters both the structure (vacuolisation) and function of mitochondria (alteration in electrons transport and Ca^{2+} loading, decreased ATP production, apoptosis, impaired axonal transport) since the early stage of the disease (Tafuri et al., 2015; Vehviläinen et al., 2014) not only within the nervous system but also at the skeletal muscle level (Vielhaber et al., 1999; Ehinger et al., 2015).

Moreover, several shreds of evidence demonstrated that also RNA misregulation occurring in ALS augments the oxidative stress within neurons. However, dual views regarding mechanism and causation have been proposed. On the one hand, oxidative stress directly causes RNA dysregulation, as demonstrated by the cytoplasmatic mislocalisation and increased aggregation of RNA binding proteins, such as FUS and TDP43, during oxidative stress (Vance et al., 2013). On the other hand, RNA dysregulation is responsible for the oxidative stress and mitochondrial damage, as demonstrated by the physiological interaction between FUS and HSP60 within mitochondria (Deng et al., 2015) and by the TDP43-mediated regulation of proteins involved in mitochondrial physiology (Wang et al., 2013b).

Additionally, mitophagy (the selective process whereby mitochondria are targeted and degraded by the autophagy machinery) is directly involved in oxidative stress and thus in ALS pathogenesis (Edens et al., 2016). The most reliable evidence supporting the contribution of impaired mitophagy to oxidative stress lies in ALS-related genes. For example, Optineurin fulfils a pivotal role in PINK1-Parkin-mediated mitophagy (Wong and Holzbaur 2014), VCP lies downstream Parkin (an E3 ubiquitin ligase) and is recruited to the outer membrane of damaged mitochondria (Kim et al., 2013b), while TBK1 is activated through a PINK1-Parkin-dependent mechanism to recruit autophagy receptors to the depolarised mitochondria (Heo et al., 2015). Thus, once mutated, these proteins are no more able to exert their physiological function affecting the mitochondrial activity and, thereby, increasing the cellular oxidative stress. The practical role and timing of oxidative stress

in ALS pathogenesis is still unclear; however, even if it represents a secondary event, it is undoubtedly involved in the propagation of cellular damage that culminates with the MN death.

1.2.2 EXCITOTOXICITY

Glutamate, the primary excitatory neurotransmitter, is synthesised in the presynaptic terminal, stored in the presynaptic vesicles and release into the cleft through a calcium-dependent mechanism. Once released in response to depolarization, glutamate binds the receptors localised on pre- and post-synaptic domain. Four different families of glutamate receptors have been identified in mammals: AMPA, kainate, NMDA and metabotropic receptors. The first three families are ionotropic, meaning that when activated they open membrane channels that allow ions (Ca^{2+}) to pass through. The metabotropic family is composed by G protein-coupled receptors (GPCR), and they exert their effects transducing the signal into the cytoplasm.

The concentration of glutamate within the synaptic cleft is finely regulated, mostly by the isoform 2 of the astroglial glutamate transporter (EAAT2/GLT1), to avoid excitotoxicity (i.e. an excessive or prolonged stimulation of glutamate receptor that leads to neuron death).

Thanks to the studies performed in ALS animal models and patients, excitotoxicity has long been suspected as a mediator of the disease (Van Damme et al., 2005; Leigh and Meldrum 1996; King et al., 2016) since it has been demonstrated an increased susceptibility of MNs to the glutamate neurotransmitter (Spencer et al., 1986; Kawahara et al., 2004).

Have been suggested several direct (over-stimulation) and indirect (over-reaction) mechanisms by which the dysregulation of the glutamatergic transmission occurs in ALS (King et al., 2016). The direct mechanisms imply that the over-stimulation of MN by the excessive glutamate concentration within the cleft might be due to a decrease clearance of the neurotransmitter or due to its increased (dysregulated) release. The decrease up-take hypothesis is supported by the observation of a reduced function of EAAT2 in ALS patients (Fray et al., 1998) and disease models (Bendotti et al., 2001); while the dysregulated glutamate release could be driven by the overactivity of the presynaptic terminal but also by altered calcium buffering mediated by the “stressed” ER (Nosyreva and Kavalali 2010). Conversely, the MN over-reaction might be mediated by an alteration of the

glutamate regulation exert by GABAergic and glycinergic interneurons (Ince et al., 1993); an impaired receptors expression, particularly the NR1 and NR2 NMDA subunits (Virgo and de Belleroche 1995; Samarasinghe et al., 1996) and the GluR2 AMPA subunit (Takuma et al., 1999); or by the intrinsic excitability (i.e. firing threshold) of motor neurons (Vucic and Kiernan 2006).

1.2.3 IMPAIRED PROTEOSTASIS

The accumulation of damaged proteins contributes to several neurodegenerative diseases, and it has also emerged as a hallmark in ALS (Soto 2003). Under normal conditions, neurons possess an efficient protein quality control machinery which can also be modulated under toxic stress (adaptive mechanism) to maintain the proteostasis (i.e. protein homeostasis) (Balch et al., 2008). However, it has been reported that neurons are particularly vulnerable to disturbances in proteostasis because they are long-lived post-mitotic cells that are not able to dilute out protein aggregates during cell divisions (Yue et al., 2009). Moreover, it has been demonstrated that the neurons ability to maintain the proteostasis declines during ageing, that might explain why the majority of the neurodegenerative diseases occurs in the adulthood (Hipp et al., 2019).

Proteostasis comprises a network of interconnected quality control processes that include (Bendotti et al., 2012; Blokhuis et al., 2013):

- ✓ Chaperones_ assist protein folding and target misfolded proteins to degradation.
- ✓ Ubiquitin proteasome pathway (UPS)_ is the principal mechanism for protein catabolism in the cell. Degradation of a protein via this pathway involves two discrete and successive steps, tagging or conjugation of the substrate protein by the covalent attachment of multiple ubiquitin molecules and the subsequent degradation of the tagged protein by the 26 S proteasome.
- ✓ ER-associated protein degradation (ERAD)_ designates a cellular pathway that targets misfolded proteins from the ER to ubiquitination and subsequent degradation in the cytosol by the proteasome.

- ✓ Autophagy-lysosome pathway_ segregate misfolded proteins in a double membrane forming an autophagosome. This vesicle is then fused with the lysosome, forming the autophagolysosome, where misfolded proteins are degraded.
- ✓ Stress granules_ are cytosolic structures composed of assembled ribonucleoproteins to stop protein translation under a variety of cellular stresses.

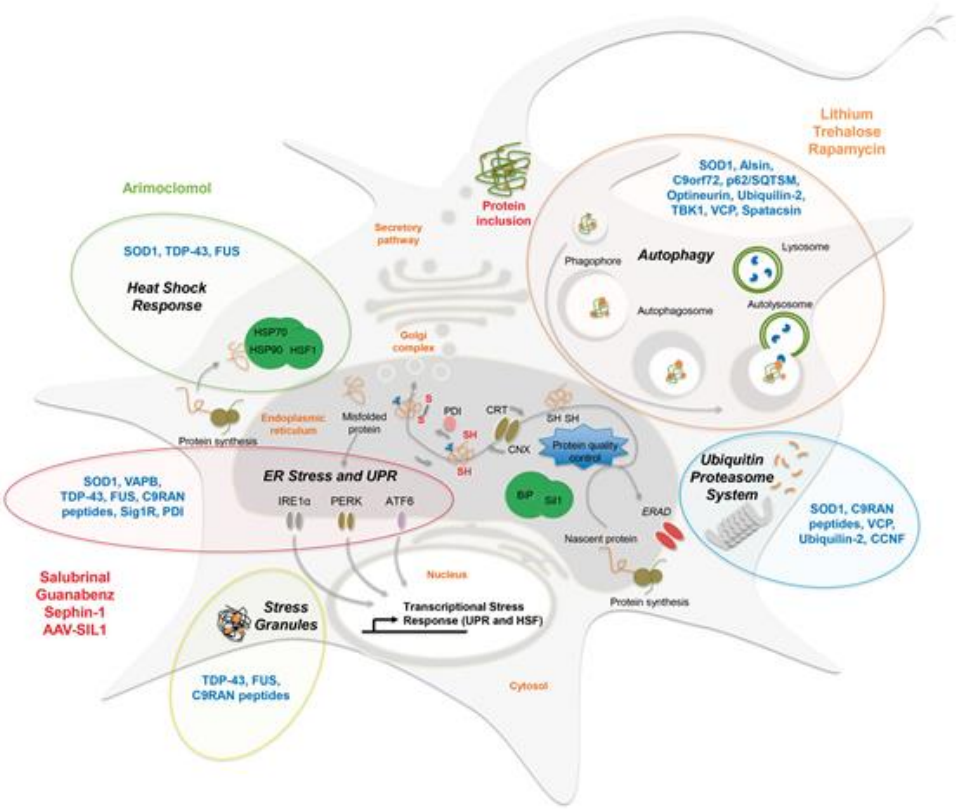

Figure 3: *Contribution to ALS-related gene to proteostasis impairment in the disease* (Medinas et al., 2017).

The most commonly ALS-related genes (*SOD1*, *C9ORF72*, *TARBDP*, *FUS*) all give rise to proteins that are involved in proteostasis machinery and found to aggregate within neurons of ALS patients (Fig. 3). Moreover, it has been recently reported that the product of mutant genes can change their native conformation and seeds in a prion-like mechanism (McAlary et al., 2019). However, proteinaceous inclusions are also observed in sALS patients, implying that disturbances in protein folding and quality control mechanisms may bring wild-type proteins to misfold and aggregate (Bosco et al., 2010; Neumann et al., 2006; Migheli et al., 1990). These pieces of evidence suggest that the impairment of the proteostasis quality control can be a common feature of both familial and sporadic ALS.

Protein aggregates have also been found in disease models, particularly within dendrites, periaxonal processes of oligodendrocytes and in neurons and astrocytes perikarya (Watanabe et al. 2001; Stieber et al., 2000). Studies performed showed that these inclusions are composed of several proteins, such as SOD1 (Bosco et al., 2010), ubiquitin (Basso et al., 2009), chaperones (Marino et al., 2015), TDP43 (Sanelli et al., 2007), Optineurin (Korac et al., 2013), neurofilaments (Beaulieu et al., 2000), and many more.

Interestingly, glial cells and muscles seem mostly spare from the misfolded protein accumulation. Recently it has been suggested that these cell types are better equipped in activating chaperones and protein degradation system than neurons, resulting in a more efficient response to counteract the altered proteostasis (Galbiati et al., 2014; Jansen et al., 2014).

In conclusion, the accumulation of ubiquitin or ubiquitin-tagged misfolded proteins could affect the physiological activity of proteasome machinery, impairing the ordinary protein degradation and establishing a vicious circle that increases the protein accumulation, thus resulting in MN degeneration and death. However, it is not yet known if the altered proteostasis in ALS is caused by an excessive protein accumulation or by an overwhelmed protein clearance.

1.2.4 AXONAL TRANSPORT DEFECT

Axonal transport involves the movement and distribution of intracellular cargo such as proteins, mRNA, vesicles, lipids and organelles along the axon. Intermediate filaments (IF) represent the “binaries” of this transport, which, together with microtubules and microfilaments, compose the eukaryotic cells cytoskeleton.

In the adult neurons, three major IF types are present: neurofilaments, α-internexin and peripherin. Thanks to these “binaries” neurons can transport protein synthesised within somata to the neuromuscular junction (kinesin-mediate anterograde transport) and substances produced in the periphery to the cell body (dynein-dynactin-mediated retrograde transport).

Neurofilaments (Nfs) are the major IF within neurons and represent the most abundant part of large myelinated axons, of which control the calibre. Nfs are composed by the polymerisation of

the light (Nf-L, 65KDa), the medium (Nf-M, 95KDa) and the heavy (Nf-H, 115KDa) subunits. Nf-L is fundamental in filament assembly, while the other two subunits links with other Nfs.

Axonal transport defects are commonly seen in many neurodegenerative diseases, most of them mimicking ALS (Guo t al., 2019). Its involvement as a pathogenetic mechanism in ALS arose from the observation of abnormal accumulation of Nfs, mitochondria and lysosome in MN perikarya (hyaline conglomerate inclusions) of *post mortem* tissues (Hirano et al., 1984). Peripherin and Nf have also been found in the majority of axonal inclusions (axonal spheroids) of ALS patients (Corbo and Hays 1992), particularly in the large calibre axons of α-MNs, which are the more susceptible to the disease (Sobue et al., 1981).

The mechanism driving the formation of Nfs aggregates in ALS is still unclear. Mutation in Nf genes have been found in fALS and sALS patients (Figlewicz et al., 1994) and seems to be correlated to post-translational protein modification, particularly hyperphosphorylation (Dale and Garcia 2012). However, Nf gene mutations are not a common cause of ALS but could represent a risk factor for MNs vulnerability (Bonafede and Mariotti 2017).

Clinical studies suggest that the Nfs aggregation could also be promoted by their altered stoichiometry (Zucchi et al., 2018), as confirmed by overexpression/downregulation studies performed in disease models (Turner and Talbot 2008). Surprisingly, the overexpression of the Nf-L and Nf-H was able to slow down the disease in ALS mice, suggesting a protective effect of neurofilaments accumulation when occurring within neuron cell body (Kong and Xu 2000).

Aside from the accumulation, Nfs can also release after neuroaxonal damage and thus can be titrated in biofluids (CSF and serum). Nf-L dosage is currently used in clinical practice as a diagnostic and informative biomarker since it has been demonstrated its increased level ~12 months preceding the manifestation of the first signs of the disease (Benatar et al. 2019; 2018). Further studies also showed its predictive value in ALS prognosis (Poesen and Van Damme 2018).

Nfs are a component of cell cytoskeleton and with that take part in several cellular processes. Consequently, an open question concerning their involvement as a cause or consequence of the neurodegenerative event in ALS is still debated. For example, it is well known that neurons are

critically dependent on mitochondria to maintain their function (Schwarz 2013). However, lack of mitochondrial adaptors or regulators (e.g. Miro, Milton, Kif5C, etc.) has been likewise connected to ALS mutation (Chen et al., 2016; Mórotz et al., 2012b) suggesting that mitochondrial deficit could be the causative reason of axonal transport deficit. Similarly, other pathogenic mechanisms such as ER stress (Woehlbier et al., 2016), protein aggregation (Oberstadt et al., 2018; Huai and Zhang 2019), autophagy dysregulation (Lie and Nixon 2019) and DNA damage (Naumann et al., 2018) are strictly associated to axonal transport deficit. Nevertheless, further studies are ongoing to clarify whether these pathways are localised upstream or downstream to axonal transport defects in ALS.

1.2.5 NUCLEOCYTOPLASMIC TRANSPORT DEFECTS

The earliest clue suggesting a nucleocytoplasmic transport defect in ALS arose from a study performed in 1995, showing lateral arthrogryposis within the anterior horn of eleven Finnish families (Vuopala et al., 1995). Later on, an analysis of a large cohort of patients leads to the identification of a rare loss of function mutation in *GLE1* (Kaneb et al., 2015), which encodes a component of the cytoplasmic face of the nuclear pore complex (NPC) that facilitates the export of mRNAs from the nucleus (Nousiainen et al., 2008). This evidence was strengthened by the nuclear depletion and cytoplasmic mislocalisation of TDP43 (Winton et al., 2008), FUS (Ling et al., 2013) and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) (Liu et al., 2016), together with the observation of a high number of mutations within the nuclear localisation signal of ALS-linked proteins. These observations suggested that dysfunction in nucleocytoplasmic transport through the NPC might contribute to the disease development and/or progression, either through the loss of nuclear functions or gain of toxic cytoplasmic function that increase concentration/residence time of these proteins in cytoplasmic assemblies (Kim and Taylor 2017).

Another link between nucleocytoplasmic transport deficit and ALS has emerged with the discovery of the G_4C_2 expansion of *C9ORF72* gene. Recent works demonstrated that several RNA binding proteins and NPC components (e.g. Nup205, Nup107) are sequestered within RNA foci and that the repeated expansion alters the nuclear-cytoplasmic distribution of TDP43 (Zhang et al., 2015a; Zhang et al., 2016). Although these studies did not distinguish between RNA-mediated and DPR-mediated

toxicity of mutant C9ORF72, Jovičić and colleagues identified eleven altered genes that regulate nucleocytoplasmic transport in a model of DPR-mediated toxicity (Jovičić et al., 2015).

1.2.6 IMPAIRED DNA REPAIR

Due to its high rate of oxygen consumption and metabolic activity, the CNS is more susceptible to DNA damage. Indeed, oxidative stress is the leading cause of DNA damage (i.e. double-strand breaks, DSBs) within neurons (Tann et al., 2011). Moreover, the high transcriptional rate also increases neurons susceptibility to injury since the genomic stability is fundamental for the maintenance of homeostasis (Aguilera and García-Muse 2012; Hill et al., 2016). However, differently from other cell types, homologous recombination (HR) is not an available mechanism for DNA repair since neurons are long-lived post-mitotic cells.

The first evidence of the impaired DNA damage response (DDR) in ALS was reported more than thirty years ago (Tandan et al., 1987). Concurrently with the discover of ALS-associated genes, considerable evidence of an altered DDR in ALS have been collected.

It has been observed that FUS and TDP43 participate in the DDR. The former physiologically colocalises with the histone protein γH2AX and RNA polymerase II (RNA pol II) at sites of damage primarily to prevent or repair loop-associated DNA damage (Hill et al., 2016). TDP43, as FUS, colocalises with RNA pol II, moreover it binds PAR (the polymer product by *PARP1*) stabilising the DNA replication forks and chromosome remodelling (Rulten et al., 2014). TDP43 is also involved in the non-homologous end-joining and base-excision repair mechanism thanks to the interaction with the histone deacetylases SIRT1 and HDAC1 (Wang et al., 2013a; Wang and Hegde 2019). All these physiological functions are lost by the mutant proteins.

More recently, it has also been demonstrated a correlation between the G_4C_2 expansion of *C9ORF72* and a defective DNA repair, manifested by higher levels of DSBs and impaired DDR (Walker et al., 2017). Consistently, high levels of DNA damage markers (p53, GADD45, γH2AX, etc.) have been observed in iPS-derived motor neurons of ALS-FTD1 patients (Lopez-Gonzalez et al., 2016).

1.2.7 ALTERED RNA METABOLISM

The interest in the involvement of RNA dysregulation in the pathogenesis of ALS arose from the identification of disease-causing variations in RNA-binding protein (RBP) genes: *FUS* and *TARDBP*. RBPs are involved in several aspects of RNA metabolism including splicing, transcription, transport, translation and storage within the stress granules (Dreyfuss et al., 2002). These abilities are mainly derived from the prion-like domain of RBPs, a site where the major part of mutations occurs. Interestingly, this domain is involved in the stress granules formation thanks to its ability to form multiple transient weak interactions with other proteins/RNA. Stress granules are RNA and proteins-made complexes formed during cell stress to transiently inhibit translation of non-essential RNA or pro-apoptotic proteins (Protter and Parker 2016). However, the involvement of stress granules formation in ALS pathogenesis is currently under investigation (Fernandes et al., 2018).

TDP43 mainly regulates the splicing, stability and transport of ~6˙000 RNA (Buratti and Baralle 2008; Polymenidou et al., 2011; Colombrita et al., 2012) which encode for synaptic proteins (GABA receptors, AMPA receptor subunit, microtubule-associated proteins, etc.) and proteins implicated in ALS and other neurodegenerative diseases (FUS, Ataxin2, etc.) (Butti and Patten 2019). Moreover, it has been demonstrated TDP43 involvement in microRNA (miRNA) biogenesis and long non-coding RNA (lncRNA) binding (Kawahara and Mieda-Sato 2012; Tollervey et al. 2011). Interestingly, TDP43 retains the ability to reduce its owns expression by bind the 3'UTR of its own pre-mRNA (Ayala et al., 2011), highlighting the importance of TDP43 in cell homeostasis and thus the significance of its deregulation/dysregulation in ALS pathogenesis.

Similarly to TDP43, FUS binds different transcript of ALS-associated genes (e.g. *VCP*, *OPTN*, *VAPB*) (Lagier-Tourenne et al., 2012), modulates the biogenesis of miRNA (Morlando et al., 2012) and transcriptional factors (e.g. NF-κB) (Uranishi et al., 2001). As part of hnRNP complex, FUS regulates the spicing of ~1˙000 mRNA (Lagier-Tourenne et al., 2012) involved in neurogenesis, cytoskeleton organisation, axonal outgrowth and maintenance, potential transmission to skeletal muscles and several others important cell functions (Butti and Patten 2019).

Although SOD1 is not an RBP, pieces of evidence demonstrate its involvement in the regulation of the metabolism of VEGF and Nf-L subunit transcripts (Lu et al., 2009; Chen et al., 2014a).

More recently, it has been observed RNA foci deposition in ALS and FDT patients characterised by the G_4C_2 expansion of *C9ORF72*, due to both sense and antisense transcription of the altered gene. RNA foci sequester several RBPs (e.g. hnRNP3, FUS, TDP43) leading to the dysregulation of the RNA metabolism (Lee et al., 2013) and thus affecting multiple cell functions including stress response, nuclear transport, synaptic transmission and cell-to-cell signalling. Moreover, also the DPR produced by the RAN (non-AUG) translation can modify the splicing patterns of several transcripts (Suzuki et al., 2018; Zhang et al., 2015a).

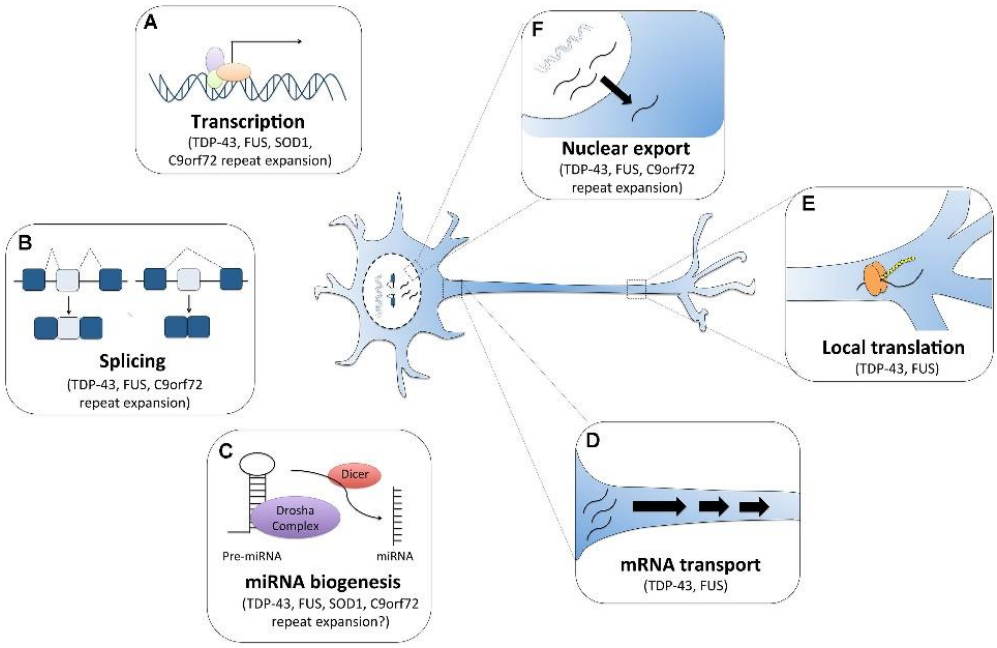

Figure 4: *Major ALS-related mutations that disrupt RNA processing through several mechanisms* (Butti and Patten 2019).

1.2.8 NON-CELL AUTONOMOUS MECHANISMS

Besides the pathogenetic mechanisms hitherto described, several pieces of evidence support the involvement of non-cell autonomous processes in ALS development and progression. Thanks to the new insights provided by the basic research studies, it has been observed that other cells types, besides MNs, such as glial cells and immune cells, actively participate to ALS pathogenesis (Thonhoff et al., 2018; Chiot et al., 2019).

Moreover, it has been demonstrated that ALS is a multisystemic disease in which the first signs appears in the peripheral compartment (i.e. muscles and nerves). Indeed, the pathological

modifications in motor axons and nerve terminals precede the MN degeneration and onset of clinical symptoms (Dadon-Nachum et al., 2011). This indication has led to ALS being suggested as a distal axonopathy (Fischer et al., 2004), whereby skeletal muscle contributes to a retrograde signalling cascade that affects MNs (Moloney et al., 2014; Dupuis et al., 2009).

The non-cell autonomous pathogenetic mechanisms of ALS are strictly correlated with the current study and, therefore, will be discussed more in detail in the next section of this Thesis.

1.3 NON-CELL AUTONOMOUS MECHANISM IN ALS

Several pieces of evidence indicate that the neurodegeneration in ALS also occurs due to the dysregulated environment surrounding MNs, which drives a cascade of events collectively known as "neuroinflammation". This phenomenon is characterised by the activation of microglia and astrocytes, infiltration of peripheral immune cells and elevated release of inflammatory mediators in the CNS (Komine and Yamanaka 2015).

The neuroinflammation in ALS, as in other neurodegenerative diseases, is characterised mainly by the innate rather than the adaptive immune response (Prinz and Priller 2017). However, while the astrogliosis is the main feature of ALS (Turner et al., 2004) that can be observed even at the pre-symptomatic stage of the disease in the rodents models (Sanagi et al., 2010), the studies performed showed the infiltration also of T lymphocytes and non-resident innate immune cells (mast cells, dendritic cells, macrophages) in the CNS of ALS patients and models (Graves et al., 2004; Engelhardt et al., 1993; Alexianu et al., 2001). Nevertheless, the role of the infiltrating immune cells and the immune-mediated response in the disease pathogenesis remains poorly understood.

Evidence of the immune and glial cells affecting the fate of MNs comes from the characterisation of the mutant SOD1 (mSOD1) transgenic mouse models of ALS. Studies performed in rodents demonstrated that the expression of human mSOD1 specifically within MN was not sufficient to induce neurodegeneration in mice (Pramatarova et al., 2001; Lino et al., 2002). Intriguingly, it has been observed that the expression of mSOD1 at very high levels within MN led to the development of the disease very late in the mouse life with a progression rate much slower than mice expressing the mSOD1 transgene ubiquitously (Jaarsma et al., 2008). In keeping with this, the specific silencing

of the human transgene in neurons delayed the disease onset, but cannot alter the disease progression of mSOD1 mice (Ralph et al. 2005).

These findings highlighted the involvement of non-neuronal cells in the pathogenesis of ALS. However, it has been observed that the selective mSOD1 expression in non-neuronal cells did not lead to the development of the disease but damaged MNs (Yamanaka et al., 2008; Gong et al., 2000; Beers et al., 2006), suggesting a pathogenic interaction between neurons and glial cells in ALS. Moreover, it has been reported that the astrocyte (Yamanaka et al., 2008) or microglia (Boillée et al., 2006b; Beers et al., 2006) specific deletion of mSOD1 significantly slowed down the disease progression, without altering the motor onset of ALS mice. Notably, the mSOD1 deletion from astrocytes was accompanied by a delay in the activation of microglia cells, suggesting the direct pathogenic crosstalk of astroglial cells in the disease (Yamanaka et al., 2008).

Other non-neuronal cells, such as the oligodendrocytes, contribute to MN damage, although through non-inflammatory mechanisms. Indeed, the removal of mSOD1 in oligodendrocyte progenitors ($NG2^+$ cells) delayed the disease onset and prolonged the survival of ALS mice (Kang et al., 2013). These results suggest that the profound loss of grey matter oligodendrocytes in ALS, and the inability to restore their function, accelerate the damage of vulnerable MNs (Kang et al., 2013).

Besides the resident non-neuronal cells, it has been reported that also the T cells and monocytes actively participates to the MN degeneration (Coque et al., 2019; Raoul et al., 2002; Butovsky et al., 2012). However, the depletion of the entire T lymphocyte population crossbreeding the mSOD1 mice with $RAG2^{-/-}$ or $TCR^{-/-}$ animals significantly worsened the disease course of the double transgenic mice (Chiu et al., 2008; Beers et al., 2008).

These pieces of evidence suggest that, despite the plethora of intrinsic mechanisms that led intracellular injury, MNs do not die alone; glia cells and immune cells are required to mediate the progression of the neurodegenerative cascade in ALS. However, the contribution of the immune response in ALS determination and progression is still under debate.

The initial belief regarding the involvement of the non-neuronal cell in ALS was that all the cellular players embraced were activated toward a neurotoxic state. This idea was argued following the

observation that wild-type (WT) glial cells, as well as the transplantation of WT bone marrow, extended the survival of mSOD1 mice (Clement et al., 2003; Corti et al., 2004; Ohnishi et al., 2009), indicating an intrinsic toxic capability of ALS immune cells. However, the passive transfer of mSOD1 $CD4^+$ T lymphocytes or $CD4^+$ $CD25^+$ T regulatory (T reg) cells harvested from mSOD1 donor mice during the first (i.e. stable) disease phase, but not WT lymphocytes, slowed down the disease in double transgenic mSOD1/$RAG2^{-/-}$ mice. Conversely, the transfer of T cells harvested from mSOD1 in the advanced disease stage did not exert the same beneficial effect (Beers et al., 2011a). These studies showed that T reg cells exerted an inductive action on glial cells promoting the polarisation toward the anti-inflammatory phenotype and release of trophic factors (Beers et al., 2008; Chiu et al., 2008), also highlighting the protective role of the non-neuronal cells in the ALS pathogenic cascade.

Therefore, it has been postulated that the neuroinflammatory event occurring during ALS might result in two distinct phases. The first phase, appearing in the initial stage of the disease, is characterised by an anti-inflammatory/neuroprotective compensatory response of glia and immune cells that release neurotrophic factors as an endeavour to decrease the MN stress. Late in the disease, as more neurons are being damaged, a second phase takes place, characterised by a cytotoxic response of glia and immune cells (Hooten et al., 2015).

The first phase is mainly governed by supportive microglia and astrocytes as well as T helper 2 (Th2) and T reg lymphocytes. Th2 and T regs cells produce high levels of Interleukin 4 (IL4), thus inducing the microglia polarisation toward the neuroprotective M2 phenotype (Chiu et al., 2008). Furthermore, neuroprotective T lymphocytes can also influence astroglia behaviour leading to the release of anti-inflammatory cytokines (e.g. IL10) and neurotrophic factors (e.g. glial-derived neurotrophic factor, GDNF; insulin-like growth factor, IGF1) (Beers et al., 2008). As neuronal damage progresses, the response of the neuroprotective T lymphocytes is suppressed, and Th1 cells produce high levels of pro-inflammatory cytokines that, together with the activating factors released by the injured neurons, activate the pathway mediated by the master control gene nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The NF-κB pathway activation fosters

the microglia M1-cytotoxic phenotype and the astrogliosis (Beers et al., 2008; 2011b). The second phase is characterised by the release of several pro-inflammatory factors, such as tumour necrosis factor α (TNFα), interferon γ (IFNγ), IL1β, transforming growth factor β (TGFβ) and monocyte chemoattractant protein 1 (MCP1) (Endo and Yamanaka 2015; Liao et al., 2012; Sargsyan et al., 2009), which accelerate the disease course. Indeed, the M1-polarised cells promote proliferation and function of Th1/Th17 lymphocytes, which release inflammatory factor, thus establishing a vicious circle that results in the propagation of the inflammation and therefore in the disease progression (Hooten et al., 2015).

This evidence highlights a tight neuro-immune dialogue during the neuroinflammatory phenomenon of ALS and that the net effect of this crosstalk critically depends on the context of the interaction. Indeed, the evidence collected shown that T lymphocytes can polarise the microglia toward the M2-neuroprotective or M1-neurotoxic phenotype depending on the T cells subtype and the cytokine milieu (Appel et al., 2010). Moreover, all cytokines-producing cell types release factors that can influence the activation state and the protective/toxic activity of the other neighbouring cells (Boillée et al., 2006a; 2006b).

The evidence collected suggests that injured MNs initiate the inflammatory response, which is propagated by the glia and immune cells. However, the exact mechanism is still unknown. Arguably the mSOD1 released by neurons activates astroglia through the toll-like receptor (TLR)-mediated response (Zhao et al., 2010). Once activated astroglia release inflammatory factors fostering the recruitment of the immune cells. Intriguingly, it has been demonstrated that mSOD1 can activate microglia to the M1-proinflammatory state but also promote the release of trophic factors thus inducing a neuroprotective phenotype (Philips and Robberecht 2011; Meissner et al., 2010; Van Damme et al., 2008; Philips et al., 2010). Accordingly, the characterisation of the transcriptional profile of microglia isolated from mSOD1 mice at different stages of the disease reported the expression of both pro- and anti-inflammatory genes at the same time (Chiu et al., 2013). These observations suggested that a highly complex mechanism occurs in ALS, in which the M1 or M2 polarization of microglia is not a mutually exclusive state. The balance between harmful and

protective phenotypes of microglia is actively influenced by both intrinsic and extrinsic factors, released by damaged MNs, astrocytes and infiltrating immune cells. Similarly, it has been reported that astrocytes isolated from pre-symptomatic mSOD1 mice downregulated the peroxisome proliferator-activated receptor 1 alpha (PCG1α), indicating an earlier impairment in the ROS detoxifying activity, and are already enriched in genes linked to toxic or apoptotic effects such as CXC-motif chemokine 10 (CXCL10) and insulin-like growth factor binding protein 7 (IGFBP7) (Sun et al., 2015).

Therefore, the preclinical evidence obtained through the characterisation of mSOD1 mice indicates that the mutant transgene expression by neuronal and non-neuronal cells is fundamental for the ALS determination and progression. In particular, the mSOD1 expression by MNs is pivotal in determining the timing of the disease onset and early progression. In contrast, its expression within resident or infiltrating non-neuronal cells can actively influence the disease severity and progression (Lyon et al., 2019).

Moreover, ALS is defined as a multisystemic disease in which structural, physiological and metabolic alteration in different cells types may act synergistically to sustain and exacerbate its course. However, the contribution of the peripheral compartment in the ALS pathogenic cascade was underestimated so far. Indeed, although mSOD1 is also expressed within ALS patients muscle, its relative contribution to the muscular damage and disease progression has been debated. A pioneering work showed that the silencing of the muscular mSOD1 was not sufficient to rescue the motor ability in $SOD1^{G93A}$ mice (Miller et al., 2006). Conversely, more recently, it has been reported that the specific expression of mSOD1 within skeletal muscle was sufficient to induce severe muscle damage (Wong and Martin 2010; Dobrowolny et al., 2008). Although the effect on astroglia activation and neurodegeneration was controversial, this evidence challenges the accepted dogma that MN degeneration, caused by the mSOD1 expression, is the primary cause of muscle atrophy in the disease.

These observations derived from the characterisation of mSOD1 mice, which mirrors an exiguous percentage of ALS cases. Therefore, the development of others disease models, such and FUS,

TDP43 or C9orf72 mice, offers a precious opportunity to test the contribution of the inflammatory phenomenon to the non-cell autonomous degeneration of MN in ALS.

In most of these models clear signs of astrogliosis were readily reproduced, confirming the involvement of astrocytes and microglia in the ALS course (Alrafiah 2018). However, data collected so far are still controversial. If, on the one hand, the expression of the $TDP43^{M337V}$ variant by astrocytes led to neurodegeneration and muscle denervation in a rat model of ALS (Tong et al., 2013), on the other, the delayed activation coupled with a gradual increase of the mutant $TDP43^{A315T}$ expression in the CNS of mature mice resulted in progressive functional deficits with neuron and muscle loss but the absence of a glial response, indicating that astrocytes are not involved in the TDP43-mediated toxicity (Chan et al., 2020). Similarly, *in vitro* experiments reported that the TDP43 knock-out or the expression of the mutant protein in astrocytes was not sufficient to induce degeneration in co-cultured MNs (Serio et al., 2013; Haidet-Phillips et al., 2013). However, although the expression of the human TDP43 gene with a defective nuclear localisation signal (hTDP43ΔNLS) affected MNs viability, minimal microglial activation was observed. Intriguingly, when the hTDP43ΔNLS was suppressed, the microglia proliferation significantly increased to clear the TDP43 aggregates, resulting in functional recovery (Spiller et al., 2018).

Conversely, the specific motoneuronal expression of the mutant FUS was sufficient to drive neurodegeneration, thereby pointing to a cell-autonomous mechanism (Sharma et al., 2016; Scekic-Zahirovic et al., 2016). However, it has been suggested that the motor symptoms could be caused by the concerted action of the mutant FUS in MNs and other cell types, including oligodendrocytes (Scekic-Zahirovic et al., 2017). According to the involvement of non-neuronal cell in the FUS-mediated toxicity, it has been recently reported that the overexpression of the mutant or WT protein within astrocytes significantly affected their reactivity and drove their properties toward pro-inflammatory and neurotoxic functions (Ajmone-Cat et al., 2019; Kia et al., 2018).

Contradictory observations have been obtained regarding the involvement of glial cells in the c9orf72 ALS models, since the astrogliosis is not a common pathological feature of the hexanucleotide repeat expansion (c9-HRE) or knock-out mice (Koppers et al., 2015; Peters et al.,

2015; Jiang et al., 2016). However, recent studies suggest that astrocytes from c9-HRE carriers with ALS can mediate neurotoxicity. Indeed, it has been shown that the partial replacement of the culture medium of murine embryonic stem cell-derived MNs co-cultured with fibroblast-derived astrocytes from c9-ALS patients with conditioned medium of control astrocyte did not prevent cell death (Meyer et al., 2014). This observation suggests the involvement of a gain-of-toxic-function mechanism (possibly impairing neuronal autophagy) rather than insufficient trophic support by the c9-HRE astrocytes in driving neuronal death (Meyer et al., 2014). Accordingly, the conditioned media of c9-HRE astrocytes was sufficient to dramatically decreased the viability of induced pluripotent stem cell (iPSC)-derived MNs of C9-ALS patients or control subjects (Madill et al., 2017). Furthermore, it has been observed that the DRP length or type and/or additional concomitant factors (e.g. TDP43 phosphorylation) significantly affect the microglial activation in c9-ALS models (Schludi et al., 2017; Zhang et al. 2016; 2018b).

Although a more in-depth characterisation of these ALS models is needed, these observations did not rule out the central and dual role of the inflammatory response in the early compared with late stage of the disease. Moreover, the scant knowledge acquired on these disease models did not allow comprehending the role of the non-resident immune cells in governing the ALS pathogenic cascade.

Notably, besides the ALS-associated gene model, mouse strains vary in their immune response (McCombe and Henderson 2011). Moreover, it has been reported that non-specific inflammation (i.e. chronic LPS administration) worsens the disease course in ALS rodents (Nguyen 2004), indicating that the immune system can exacerbate the disease in the murine models independently from the primary toxic insult (i.e. mSOD1). Therefore, the observation obtained from mSOD1 mice and other ALS models cannot necessarily be generalised to patients since human ALS suffers have different immune system capability, which could be responsible for the high phenotypic heterogeneity (Ticozzi and Silani 2018; Bendotti et al., 2020). Indeed, a recent study of gene expression showed that ALS patients could be divided into two subgroups: patients with higher expression of IL6R and myeloid lineage-specific genes, and patients with higher expression of IL23a

and lymphoid-specific genes (Swindell et al., 2019). Although an immunological component to ALS pathophysiology has also been recognised (Malaspina et al., 2015) and prior studies have identified alterations in immune cell abundance and activity in ALS patients (Murdock et al., 2017), recent pieces of evidence suggest a different contribution of the inflammation in the PNS compared with the CNS (Dibaj et al., 2011; Thonhoff, Simpson, and Appel 2018). These observations elicited a fervent debate concerning the role of the inflammatory response in the peripheral compartment of ALS. Indeed, recent evidence suggested that the inflammatory phenomenon and the related infiltration of immune cells could aid in the response of the peripheral axon to degeneration (Kano et al. 2012; Dibaj et al. 2011; Schreiber et al. 2019; Nardo et al., 2016b).

The knowledge so far acquired demonstrated a pivotal role of non-neuronal resident or infiltrating immune cells in ALS. However, their temporal (early or late in the disease) or spatial (CNS versus peripheral compartment) contribution in the disease determination and progression is still unclear.

1.3.1 INNATE IMMUNITY

Innate immunity is an antigen-independent response used by the host to defend itself from an intruding pathogen immediately. The mechanisms of defence triggered by innate immunity are not specific, and it does not have any immunologic memory.

The innate system relies on pattern recognition receptors (PRRs) to respond to pathogens, which are recognised thanks to the presence of pathogen-associated molecular patterns (PAMPS). The essential function of innate immunity is to recruit immune cells to the site of infection/damage and to trigger inflammation through the production of cytokines and chemokines (Murphy and Weaver 2016).

In the CNS, the presence of the blood brain barrier (BBB) and the blood spinal cord barrier (BSCB) allows the maintenance of the homeostasis, regulating fluctuations in electrolytes, and the passage of hormones and metabolites making it an immunologically privileged area, with limited capacity to recruit immune cells from the circulation (Pachter et al., 2003; Engelhardt and Coisne 2011). Moreover, the CNS displays low immune surveillance and the absence of specialised antigen-presenting cells, which further limit the local immune responses. Despite this “immunologically

privileged status", T lymphocytes and monocytes can be trafficked into the CNS parenchyma to instrument a specific inflammatory reaction upon tissue damage (Brown and Al-Chalabi 2017; Chiu et al. 2009; Engelhardt et al. 1993).

1.3.1.1 MICROGLIA

In the CNS, microglia are the primary myeloid cell type (Lyck et al., 2009) and are responsible for pivotal functions, such as development, immune surveillance and tissue homeostasis (Kreutzberg 1996; Matcovitch-Natan et al. 2016; Stevens and Schafer 2018).

Microglia are primarily considered as the CNS resident immune cell, which has been classically described to exist in two states: resting and activated (Cherry et al., 2014). However, two-photon imaging of healthy adult brains showed that the so-called "resting" microglia is a highly dynamic population (Nimmerjahn 2005) which actively screen their microenvironment with motile processes, exerting a crucial role in maintaining homeostasis secreting factors that allow the close communication with astrocytes and neurons (Luo and Chen 2012). Although during the steady-state these cells constantly surveying the environment, upon injury microglia migrate toward the damaged area and produce cytokines and trophic factors to mitigate the damage (Kreutzberg 1996). Through phagocytosis, microglia remove pathogens and debris, as well as regulate the synaptic pruning during the development or disease (Hong et al., 2016). As a stereotyped response to injury, microglia change their morphology and upregulate ionised calcium-binding adapter molecule 1 (Iba1) and CD11b, and gain the expression of molecules associated with antigen presentation, such as major histocompatibility complex (MHC), CD80, and CD86 which are absent in naïve microglia. Microglia finally lose their ramified morphology and surveillance mode and convert to amoeboid-like, functional cells (Kettenmann et al., 2011).

Microglia possess specific tools to react to the changes occurring within the parenchyma properly. They express toll-like receptors (TLR) and pattern recognition receptors (PPR), such as CX3CR1 (fractalkine receptor), CD200 receptor (CD200R) and triggering receptor expressed on myeloid cell 2 (TREM2). Membrane-bound fractalkine is pivotal to maintain microglia in the homeostatic non-

activated state (Cardona et al., 2006), while TREM2 is necessary not only for pathogen recognition but also for the internalisation of misfolded protein (Hsieh et al., 2009).

Some genes expressed by microglia have been suspected as causative or modifiers of ALS. Increased expression of Trem2 transcript was observed in pre-symptomatic $SOD1^{G93A}$ mice, and the p.R47H variant represents a risk factor for sporadic ALS (Cady et al., 2014). Moreover, microglia are the only cells within the CNS expressing CX3CR1, making this pathway fundamental for MN-microglia crosstalk (Harrison et al., 1998). Indeed, the deletion of CX3CR1 in $SOD1^{G93A}$ mice is associated with faster disease progression and increased MN loss (Liu et al., 2019a). Similarly, specific variants in the *cx3cr1* gene are associated with a faster ALS progression in patients, although they do not represent a risk factor (Calvo et al., 2018).

It has been reported that injured MNs and astrocytes release misfolded proteins (e.g. mSOD1) which activate microglia through CD14, TLR2, TLR4 and scavenger receptor-dependent pathways (Roberts et al., 2013; Zhao et al., 2013). Moreover, dying and degenerating neurons release ATP, which interacts with the ionotropic P2X and metabotropic P2Y purinergic receptors activating microglia (Volonté et al., 2016). This observation was confirmed in ALS patients and $SOD1^{G93A}$ mice through positron emission tomography (PET) imaging, which demonstrated a widespread activation of microglia since the early stage of the disease (Turner et al., 2004; Gargiulo et al., 2016). Interestingly, these studies reported a significant correlation between the extent of microglia activation and the severity in the symptoms manifestation (Turner et al., 2004). Notably, it has been reported that mice homozygously expressing high levels of mSOD1 specifically within MNs presented a strong microgliosis, confirming that microglia activation is strictly governed by signals released from damaged neurons (Jaarsma et al., 2008). Therefore, as the disease progresses, microglia cells acquire a cytotoxic phenotype and play a deleterious role in promoting neurodegeneration, thus governing the speed of the ALS progression.

Once activated microglia display a very distinct and different phenotype, which can be protective or toxic to MNs depending on the stage and rate of the disease progression. Once activated,

microglia cells can be divided into “classically” activated M1 microglia and “alternatively” activated M2 microglia.

M1 microglia are cytotoxic due to the release of reactive oxygen species (ROS) and pro-inflammatory factors (e.g. IL1β, TNFα, etc.). The role of cytotoxic microglia in ALS has been so far investigated. *In vitro* studies demonstrated that mSOD1 microglia were more reactive compared to wild-type cells to the pro-inflammatory LPS stimulus, resulting in the release of a plethora of neurotoxic factors thus increasing motor neurons death (Xiao et al., 2007). Accordingly, it has been reported that TLR4 antagonists efficiently protected MNs from LPS-induced lethality in spinal cord cultures, inhibiting IL1β production by stimulated microglia (De Paola et al. 2016). Using transgenic models of ALS, it has been shown that the replacing of the mSOD1 with the wild-type microglia significantly protected MNs and increased the survival of mice (Beers et al., 2006). Similarly, the removing of mSOD1 from microglia through a Cre-LoxP approach delayed the disease progression of ALS models (Wang et al. 2009; Boillée et al.,2006a).

Conversely, M2-polarised microglia produce high levels of neurotrophic (BDNF, IGF1) and anti-inflammatory (IL4, IL10) factors promoting tissue repair, extracellular matrix reconstruction and neuron survival (Zhao et al., 2013). Accordingly, the CNS delivery of IL4 in $SOD1^{G93A}$ mice resulted in a general amelioration of clinical outcomes during the early phase of the disease through the induction of M2 gene expression by microglia. However, such approach did not revert the neurodegenerative processes occurring in the late and fast progressing phase of the disease, confirming the concerted action of MNs and non-neuronal cells in ALS progression (Rossi et al. 2018).

It has been demonstrated a dual phenotypic and functional characterisation of microglia over ALS progression. During the early stage of the disease, microglia exhibit an anti-inflammatory phenotype characterised by the upregulation of CD206 and chitinase-like protein 3 (Chil3/Ym1). The M2-polarised microglia react to the initial injury releasing neuroprotective factor to promote repair and regeneration (Appel, Beers, and Henkel 2010). As the disease progresses, the “danger signals” released from damaged neurons induce microglia to release ROS and inflammatory factors

promoting their phenotypic switch toward the M1 polarisation. Accordingly, an *in vitro* paradigm showed that early-stage M2 microglia enhanced the survival of co-cultured MNs whilst late-stage M1 microglia was cytotoxic (Liao et al. 2012).

However, the M1/M2 polarisation is a more complex continuum, and microglial reactivity is multifactorial and injury-specific (Ransohoff 2016). Indeed, the coexistence of the two opposite phenotypes, more than the transition from M2 to M1 phenotype, during ALS progression has been recently suggested. The analysis of the transcriptome of mSOD1 microglia evidenced that the activation of genes involved in anti-inflammatory pathways (*Igf1, Progranulin and Trem2*) coexists with the upregulation of genes related to potentially neurotoxic factors (*Matrix metalloproteinase-12, il1β, tnfα*) (Chiu et al. 2013). Accordingly, it has been reported a parallel increase of the inducible nitric oxide synthase (iNOS; M1 marker) and arginase 1 (Arg1; M2 marker) in microglia of mSOD1 mice (Lewis et al., 2014).

As discussed above, the knowledge so far acquired suggests that, although MNs represent the site of onset, ALS is a non-cell autonomous disease in which the glial response significantly contributes to governing its progression. Therefore, targeting the microglia has been the focus of neuroprotective strategies. Pioneer studies performed showed that the administration of minocycline, a tetracycline antibiotic that prevents microglial activation, in pre-symptomatic mSOD1 mice attenuated microglial activation and ameliorated the disease progression (Kriz et al., 2002). However, its administration after the disease onset failed to exert a beneficial effect, even increasing the microgliosis (Keller et al., 2011). Interestingly, it has been reported that minocycline specifically attenuated the M1 phenotype, without influencing the expression of M2 markers (Kobayashi et al., 2013). However, the genetic deletion of pro-inflammatory cytokines did not ameliorate the disease course of ALS mice (Gowing et al., 2006). These pieces of evidence suggest the inefficiency of the specific targeting of pro-inflammatory factors, highlighting the importance of M1/M2 balance to attempt to modulate ALS progression.

Moreover, it has been shown that the deletion of the cystine/glutamate-antiporter xCT/Slc7a11 (xCT), a critical glial transporter system involved in the excessive glutamate release from M1

microglia, early in the disease of mSOD1 mice increased the expression of IL1β and concurrently reduced the M2 marker Ym1, thus resulting in anticipation of the motor impairment. Conversely, the absence of xCT in the advance stage increased Ym1 and Arg1 expression, which possibly sustained the delay of disease progression (Mesci et al., 2015).

This evidence confirms microglia as an attractive candidate to interfere with the progressive neurodegeneration occurring in ALS. However, the evidence hitherto collected suggested that, although the modulation of microglia polarisation may still be an effective strategy to counteract the neurodegeneration, the interception of other pathogenic mechanisms is necessary to obtain a significant effect on ALS course (Frakes et al. 2017; Geloso et al. 2017).

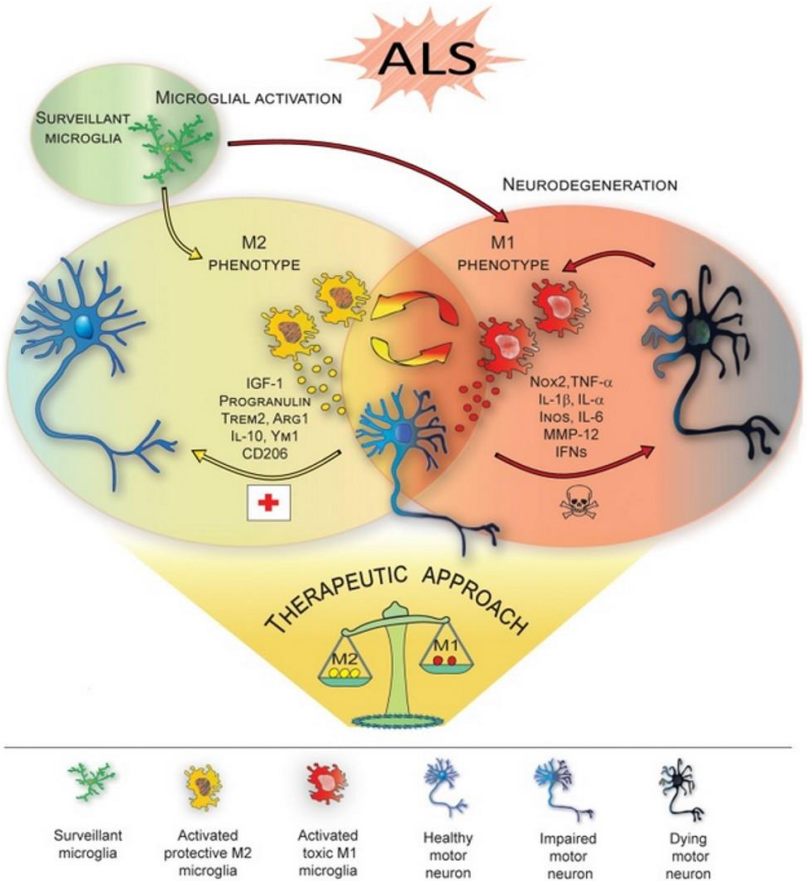

Figure 5: *M1/M2 microglia polarisation during ALS. During the disease progression activated microglia represent a continuum between the neuroprotective M2 phenotype, which promotes tissue repair and supports neuron survival by releasing neuroprotective factors, versus the toxic M1, which produces cytokines increasing inflammation and further supporting M1 polarisation, thus contributing to neuronal death* (Geloso et al. 2017).

1.3.1.2 ASTROCYTES

In the CNS, astrocytes represent the most abundant cell population. A single astrocyte can enwrap more than one million synapses and have one process with end-feet surrounding a blood vessel. This particular arrangement places astrocytes in a very suitable position to provide structural, metabolic and trophic support to neurons (Burda and Sofroniew 2014). Moreover, astrocytes are a reservoir of glycogen (Calì et al., 2019) and, thanks to the control of ionic and osmotic homeostasis, they are key players in the global and regional management of brain blood flow in response to neuronal activity (Koehler et al., 2009).

During neurodegeneration, astrocytes undergo morphological and functional modifications and respond to various stimuli, e.g. inflammatory factors produced by microglia cells (Liddelow et al., 2017). Activated astrocytes are hypertrophic, proliferate and release of pro- and anti-inflammatory cytokines and growth factors, together with components of extracellular matrix (Zamanian et al., 2012). The astrocyte activation is fundamental to limit the spread of the lesion and hamper the ongoing inflammation by preventing the infiltration of activated immune cells (Faulkner 2004). However, the resulting modification of the extracellular matrix due to the formation of the glial scar contributes to the inhibition of axonal regeneration and growth (Zamanian et al., 2012).

In ALS patients astrogliosis occurs more diffusely than microgliosis, and it is detectable in the spinal cord as well as in the grey and subcortical white matter (Nagy et al., 1994; Schiffer et al., 1996). In mSOD1 mice, reactive astrocytes appear concomitantly with a decreased number of MNs (Schiffer et al., 1996). Studies performed in ALS rodent models showed that activated astrocytes are hypertrophic and express markers that are typical for astrogliosis as well as features of immature astrocytes, such as glial fibrillary acidic protein (GFAP) (Lepore et al., 2008), connexin 43 (Cx43) (Almad et al., 2016) and the α2 subunit of Na^+/K^+ ATPase (Gallardo et al., 2014). The latter directly interacts with astrocytic glutamate transporters affecting the buffering activity of EAAT1 and EAAT2, thus altering the electrochemical gradient (Illarionava et al., 2014).

Glutamate buffering is one physiological feature of the astrocyte, fundamental to protect neurons from excitotoxicity-mediated cell death. Although it has been reported that, during the

development, the expression of EAAT2 in glia-restricted progenitors is higher in SOD1^{G93A} mice, as the disease progresses transgenic mice lose the majority of glutamate transporters (Lepore et al., 2008; Haidet-Phillips et al., 2015). Moreover, it has been shown that EAAT2 sumoylated fragments produced by caspase 3 activity accumulate within the cell nucleus causing morphological changes and dysregulating the astrocyte gene expression programme (Gibb et al., 2007), particularly those related to mitochondrial functions and cellular respiration (Foran et al., 2011). As a consequence, damaged mitochondria lose the ability to buffer intracellular Ca^{2+} and its cytosolic concentration increases, together with the concentration of ROS, NOX2 (gp91^{PHOX}) and iNOS (Agarwal et al., 2017). Consistently, the overexpression of Nrf2, a transcription factor for antioxidant genes, specifically within astrocytes conferred neuroprotection in mSOD1 mice (Vargas et al., 2008). This evidence suggests that activated astrocytes fail to support and protect MNs in ALS (Liddelow et al., 2017).

Astrocytosis is not the first event in the ALS pathogenic cascade (Schiffer et al., 1996). Nevertheless, astrocytes actively participate in the MN degeneration in a non-cell autonomous mechanism. *In vitro* and *in vivo* studies have shown that astrocytes expressing mSOD1 exert a cytotoxic effect to both wild-type and mSOD1 MNs (Di Giorgio et al., 2008; Nagai et al., 2007). Accordingly, the selective silencing of the mutant SOD1 or the transplantation of healthy astrocytes reduced MN death ameliorating the disease progression in mSOD1 mice (Lepore et al. 2008; Haidet-Phillips et al. 2011; Wang et al., 2011). Conversely, the transplantation of astrocytes expressing mSOD1 in wild-type rats induces focal MN degeneration (Papadeas et al., 2011). Intriguingly, it has been recently shown that both MNs and non-neuronal cells degenerate following the transplantation of spinal neural progenitors derived from sporadic ALS patients in the spinal cord of severe combined immunodeficient (SCID) mice, also affecting their locomotor ability (Qian et al. 2017). This process is mediated by astrocyte-specific soluble factors which, besides affecting the functioning of other cell types (e.g. microglia) and regulating their immunological responses, directly mediate the MN degeneration (Alami et al., 2018). Indeed, ALS astrocytes release several cytotoxic factors (IL6, CXCL1, CXCL10, TNFα, IFNγ, Sonic hedgehog and its responsive gene, etc.) (Bruijn et al., 1997; Diaz-Amarilla et al., 2011; Huang et al., 2014; Kia et al., 2018). Among them, the astrocytes-derived

TGFβ1 is a negative regulator of the neuroprotective anti-inflammatory response activated by microglia and T lymphocytes in the early stage of the disease (Endo and Yamanaka 2015).

Understanding the involvement of astrocytes in the neurodegenerative phenomenon and their interaction with neuronal and non-neuronal cells provides a conceptual framework that highlights the potential of this cell subtype as the focus of therapeutic effort. Indeed, the crucial role of astrocytes in ALS suggests that therapies aimed at modulating astrocytes biology may contribute to the development of integral therapeutic approaches to halt the disease progression (Pehar et al. 2018).

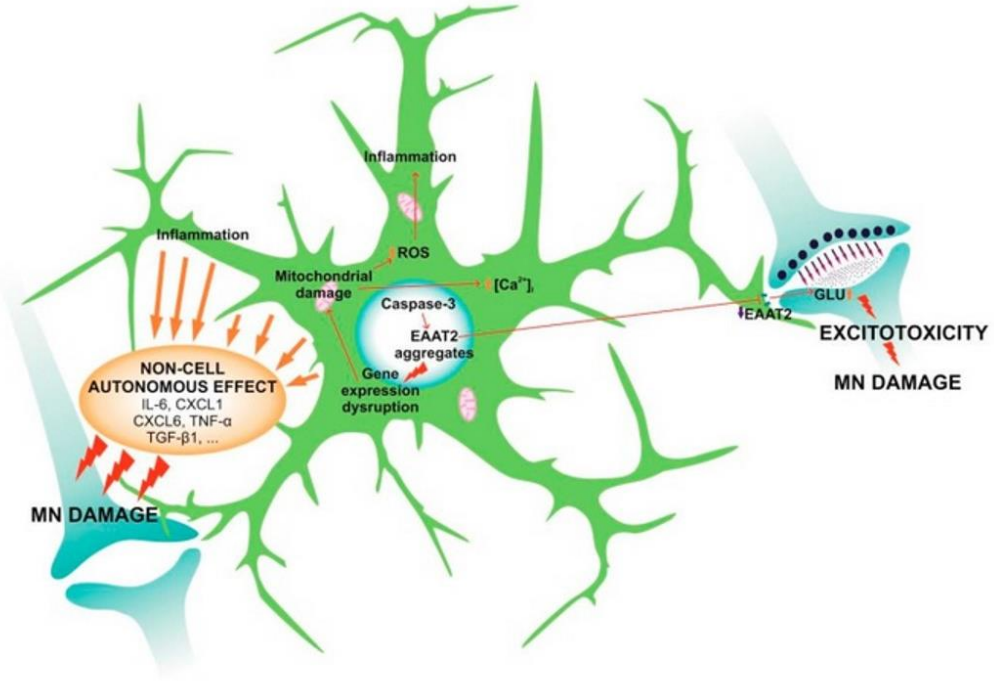

Figure 6: *Pathological changes of astrocytes during ALS* (Filipi et al. 2020).

1.3.1.3 MONOCYTES / MACROPHAGES

Monocytes constitute one component of the "mononuclear phagocyte system" (MPS), which they share with macrophages and conventional dendritic cells (cDCs) (Guilliams et al. 2014).

Monocytes develop in the adult bone marrow from a dividing common myeloid progenitor (CMP) shared with erythrocytes, cDCs, platelets and granulocytes. Following their generation, monocytes are released into the blood circulation where make up ~4% of peripheral leucocytes in mice and ~10% in humans.

Classically, monocytes were considered as a bridge linking the bone marrow-precursors with terminally differentiated macrophages and cDCs in tissues (van Furth and Cohn 1968). However, it has now become clear that in most tissues the majority of resident macrophages have an embryonic origin (Ginhoux and Guilliams 2016). These tissue-resident macrophages (e.g. microglia in the CNS) arise from a precursor in the yolk sac and then from foetal liver monocytes, which migrate to different organs (Sorokin et al., 1992). Once in the tissue, the population of these tissue-specific macrophages is maintained by self-renewal (Sieweke and Allen 2013).

In mammals, circulating monocytes can be classified in two types: the "patrolling monocytes", which have endothelial supporting functions (Auffray et al., 2007), and the "infiltrating monocytes", which possess the capability to transmigrate across the endothelium and enter in the tissue in response to appropriate signals (chemotactic gradient) (Jakubzick et al., 2013).

In humans, discrete populations of monocytes were first identified by morphology and differential expression of CD14 and CD16 (Passlick et al., 1989). The combination of these clusters of differentiation on HLA-DR$^+$ cells enabled the classification of three main subsets: CD14$^+$ CD16$^-$ cells, also known as "classical pro-inflammatory monocytes", that constitute the 80-90% of the human monocytes pool with the remaining 10-20% shared by CD14$^+$ CD16$^+$ intermediate cells and CD14^{low} CD16$^+$ "non-classical monocyte". In mice, monocytes expressing high levels of the lymphocyte antigen 6 complex (Ly6c^{hi} monocytes) have pro-inflammatory functions and show high levels of C-C chemokine receptor 2 (CCR2) and low levels of CX3C chemokine receptor 1 (CX_3CR1) (Ly6c^{high} CX_3CR1^{int} CCR2$^+$) (Geissmannet al., 2003). The Ly6c^{hi} monocytes transport antigens to the lymph node and accumulate at sites of inflammation, where they can differentiate into macrophages or dendritic cells depending on the local cytokine environment (Crane et al., 2014; Patsalos et al., 2017; Jakubzick et al., 2013). Ly6c^{low} monocytes survey the vasculature (patrolling monocytes) and are involved with tissue repair. These alternative monocytes express high levels of CX3CR1 and low levels of CCR2 (CX3CR1^{hi}CCR2^{low}) (Geissmann et al., 2003; Jakubzick et al., 2013). Gene expression analysis correlated the Ly6c^{high} and Ly6c^{low} murine monocytes with the "classical" CD14$^+$ CD16$^-$ and "non-classical" CD14^{low} CD16$^+$ human cells, respectively.

Several factors are involved in the regulation of monocytes development and differentiation. Studies in mice deficient in the colony-stimulating factor 1 receptor (MCSF/CD115) or CCR2 showed a reduced number of circulating monocytes and their retainment within the bone marrow (Tsou et al., 2007; Dai et al., 2002), indicating these factors as pivotal in monocyte mobilisation. Moreover, it has been reported that, once released in the blood under healthy homeostasis, Ly6c^{high} monocytes remain in the circulation or repopulate a proportion of tissue-resident macrophages in several organs (e.g. heart, dermis, lung) (Epelman et al., 2014; Tamoutounour et al., 2012; Jakubzick et al., 2013). An exception is the CNS, where the combination of the high self-renewal potential of the tissue-resident macrophages (i.e. microglia cells) with the restricted access because of the presence of the BBB limits their infiltration (Ajami et al., 2007; Mildner et al., 2007).

The homeostatic process of monocyte infiltration is maintained over time and requires significant gene modifications to allow the adaptation of monocyte-derived macrophages to the local tissue environment through the acquirement of a transcriptomic signature similar to whose of the resident macrophages of embryonic origin (Lavin et al. 2014; T'Jonck et al., 2018). Conversely, monocytes-derived macrophages that infiltrate during phlogosis show distinct gene signature and might respond differently to inflammation compared to embryo-derived resident macrophages (Bennett et al., 2018; Cronk et al., 2018). Moreover, they often fail to self-maintain for prolonged periods. Indeed, a low-grade tonic inflammation is required for the continuous monocytes recruitment suggesting a fine regulation of the mobilisation of the immune cells upon injury (Guilliams, Mildner, and Yona 2018). Notably, even though they can adopt a tissue-resident macrophage signature, Ly6C^{high} monocytes can also act as a local reservoir maintaining their monocyte-like state, avoiding the complete differentiation towards macrophages (Swirski et al., 2009).

An alternative maturation route of the infiltrating Ly6c^{high} monocytes is the transition into Ly6c^{low} monocytes. Non-classical monocytes exhibit an increased lifespan (~2 weeks) compared with their Ly6c^{high} counterpart ensuring the constant cell numbers even under pathological conditions, when the majority of classical monocytes are recruited to peripheral inflammatory lesions or when their

functional transition into non-classical monocytes is blocked (e.g. chronic phlogosis) (Guilliams et al., 2018). Although several factors have been shown to modulate the classical to non-classical monocytes transition (e.g. delta-like 1, C/EBPβ, NR4A1, and KLF2) (Mildner et al., 2017; Hanna et al., 2011; Gamrekelashvili et al., 2016), the exact mechanism is still unknown. Indeed, although this evidence suggests that Ly6c^{high} and Ly6c^{low} monocytes are biologically interconnected, this does not exclude that some cells in the non-classical monocyte pool might develop without passing through a classical monocyte stage (Carlin et al., 2013).

In conclusion, the leading characteristic that differentiates monocytes from others MPS members is their aptitude to be rapidly mobilised towards inflamed body compartment, where they exhibit a proinflammatory or resolving capability shaped by micro-environmental and spatial cues (Guilliams et al., 2018). However, in pathological conditions, the distinction between tissue-resident and recruited macrophages has not yet possible (Italiani and Boraschi 2014).

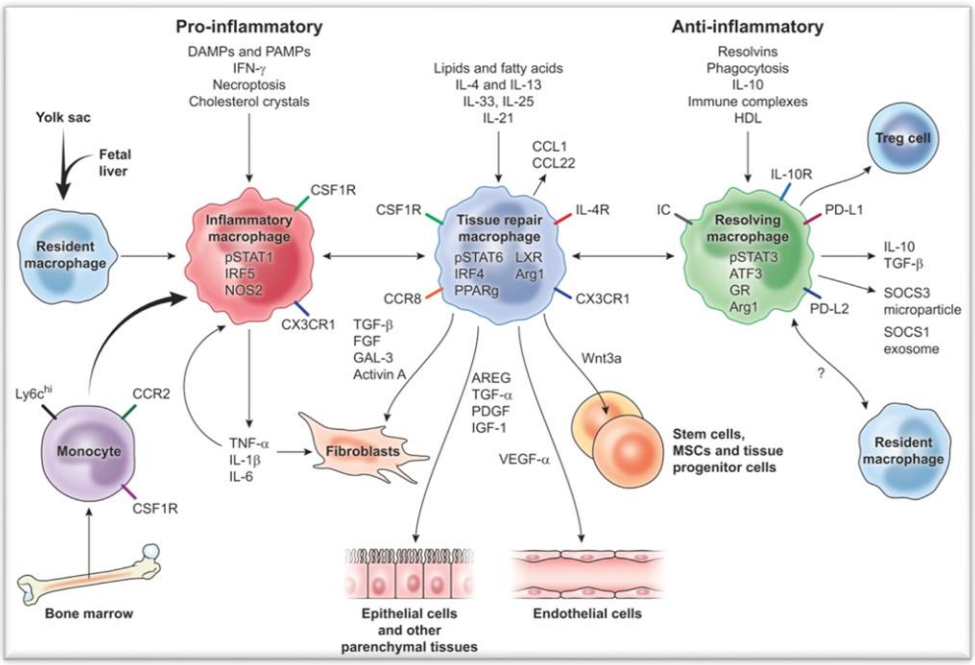

Figure 7: *In many tissues, the tissue-resident macrophage population is derived from the yolk sac and foetal liver during development but is complemented by inflammatory monocytes recruited from the bone marrow after injury. The recruited and resident macrophages undergo marked phenotypic and functional changes in response to DAMPs, PAMPs, growth factors, cytokines, and other mediators released in the local tissue microenvironment. The dominant phenotypic variants depicted here regulate inflammation, tissue repair, regeneration, and resolution. Macrophages produce a variety of factors that stimulate the proliferation, differentiation, and activation of fibroblasts, epithelial cells, endothelial cells, and stem and progenitor cells that facilitate tissue repair. During the later stages of the repair process, they assume a regulatory pro-resolving phenotype that ensures that the tissue-damaging inflammatory response is suppressed and normal tissue architecture is restored. If the process is not controlled effectively, persistent inflammation and/or maladaptive repair processes can lead to tissue-destructive fibrosis. In some cases, the recruited monocytes seed the tissues and adopt a resident macrophage phenotype (Modified from* Wynn & Vannella, 2016*).*

Traditionally, macrophages can assume both the M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotype. Macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are the primary cytokines that stimulate the macrophage M2 or M1 polarization, respectively (Hamilton 2008). Monocytes stimulated by GM-CSF participate in the antigen presentation and produce pro-inflammatory factors, such as IL12, IL23, IL1β and TNFα, amplifying the ongoing inflammation through the release also of chemoattractant factors. Moreover, the M1-polarised monocyte-derived macrophages express high levels of iNOS, thus generating NO, which can tag cellular debris for removal by phagocytosis or contribute to cytolysis of neighbouring cells (Hibbs et al., 1988). Moreover, the M1-polarised macrophages promote the Th1 response sustaining the establishment of a pro-inflammatory milieu (Martinez et al., 2008). In contrast, M-CSF-induced cells are more involved in scavenging and release anti-inflammatory cytokine (e.g.IL10, Ym1) that deactivate M1 cells supporting Th2 effector functions (Fleetwood et al., 2007; Martinez et al., 2008). Besides, the M2 macrophage-derived TGFβ can also induce arginase 1 expression (Arg 1) (Mills et al., 2000), an enzyme involved in arginine metabolism from which derive polyamines that can stimulate fibroblast proliferation and increase proline synthesis, thus leading to increased connective tissue production and tissue healing (Modolell et al., 1995).

These pieces of evidence indicate that monocytes and macrophages are versatile and plastic cells, whose function and phenotype are regulated by signals deriving from the local milieu. Although following tissue alteration pro-inflammatory M1 monocytes are recruited into the damaged area and mature to (pro-inflammatory) macrophages, this categorisation is somewhat prejudicial since these cells actively contribute to the resolution of the inflammatory response and help to restore the tissue homeostasis. Similarly to microglia cells (Chiu et al., 2013; Lewis et al., 2014), the ly6C^{high} are generally considered pro-inflammatory (M1) monocytes while the ly6C^{low} cells are the anti-inflammatory (M2) counterpart. Nevertheless, in real ongoing inflammation, infiltrating monocytes are highly heterogeneous and express mixed batches of M1/M2-associated genes as they go through the various maturation stages (Kratofil et al., 2017; Dal-Secco et al., 2015; Sica and Mantovani 2012).

The involvement of monocyte in the CNS pathology during ALS is still under debate. Assessment of circulating monocytes in ALS patients has been performed to gain insight into potential inflammatory or immune system activation in the disease. These studies showed a functional alteration of peripheral monocytes in ALS patients (Zhang et al., 2005; Nardo et al., 2011; Zondler et al., 2016), indicating their skewing toward a pro-inflammatory state (Zhao et al., 2017a).

Speculations about the mechanisms behind the monocytic dysregulation in ALS are altered bone marrow egression, different transdifferentiation between monocyte subtypes, or differential changes in tissue infiltration that results in an altered composition of the haematogenous monocytes (Zondler et al., 2016). Interestingly, it has been found a direct correlation between the levels of circulating $CD14^{+}CD16^{-}$ pro-inflammatory monocyte and the functional rating score in ALS (Murdock et al., 2016). Accordingly, it has been observed that once isolated and *in vitro* stimulated, ALS monocytes exhibit an increased ability to be activated to toxic M1 macrophages compared with immune cells derived from healthy subjects (Du et al., 2020). Besides, circulating $CD14^{+}$ monocytes are decreased in the blood of ALS patients at the early stage of the disease, indicating a potential mobilisation toward the CNS (Mantovani et al., 2009) and inflamed/damaged tissues.

It has been postulated that microglia release the pro-inflammatory chemokine monocyte chemoattractant protein 1 (MCP1) (Butovsky et al., 2012), which is elevated in the CSF of ALS patients (Nagata et al., 2007), to promote the recruitment of $CCR2^{+}$ proinflammatory monocytes (Mildner et al., 2009), possibly aggravating the MN degeneration. Properly, preclinical studies demonstrated that blocking the monocytes infiltration in the CNS resulted in the amelioration of the disease course of mSOD1 mice (Butovsky et al., 2012). However, it has been recently reported that the presence of peripheral myeloid cells in the spinal cord protected MNs from degeneration indicating that, differently from other neurodegenerative diseases (Gao et al., 2015), the monocyte infiltration in ALS might not be mediated by CD95 ligand (FasL) (Zondler et al., 2016). This evidence suggests that the role of monocytes in ALS may be highly dependent on the context of a specific neurodegenerative condition, but also on different time frames during the disease progression (Zondler et al., 2016). However, whether peripheral monocytes display an increased CNS invasion

in mSOD1 mice is still a matter of debate. Indeed several preclinical studies reported undetectable levels of monocytes-derived macrophages in the spinal cord of ALS mice (Ajami et al., 2007; Chiu et al., 2009, Chiot et al., 2020) or found them confined to the perivascular spaces of the blood brain barrier (Lewis et al., 2009). This evidence was confirmed through an in-depth RNA sequencing analysis of the myeloid population of the spinal cord of ALS mice revealing that only a negligible population of infiltrating myeloid cells was recruited during the disease progression (Chiu et al., 2013). Moreover, it has been shown that the choroid plexus of mSOD1 is not enabled to support leucocytes trafficking during the disease progression and requires to be activated (e.g. immunisation with myelin-derived peptide) to sustain the accumulation of immune cells (Kunis et al., 2015).

CNS invasion might not be necessary or at least not be the only mechanism of action mediating the impact of peripheral monocytes on the ALS course. Pre-clinical evidence recently described the involvement of monocyte-derived macrophages at the peripheral level (i.e. nerves and muscles). Studies performed in ALS mice showed that peripheral macrophages are detectable alongside degenerating nerve fibres since the pre-symptomatic stage of the disease and continue to increase until the terminal phase of the disease (Chiu et al., 2009; Graber et al., 2010; Lincecum et al., 2010; Dal Canto and Gurney 1994; Kano et al., 2012). Similarly, immune cells infiltration has been reported within the skeletal muscles of ALS rodent models (Wang et al., 2017; Trias et al., 2018; Chiu et al., 2009; Vallarola et al., 2018; Chiot et al., 2020). Accordingly, the release of chemoattractant factors (e.g. MCP1) and complement deposition were detected in the sciatic nerve and skeletal muscle of ALS mice concomitant with macrophages infiltration, indicating putative signalling through which immune cells are recruited within degenerating tissues (Chiu et al., 2009; Nardo et al., 2016b; Wang et al., 2017; Kano et al., 2012). However, it remains to be determined whether infiltrated macrophages are protective supporting tissues regeneration or serve only to remove cellular debris and amplify the ongoing inflammation.

Notably, the robust macrophages activation recorded within the nerves of $SOD1^{G93A}$ mice was not correlated with the expression of inflammatory cytokines (Chiu et al., 2009) suggesting that the

peripheral nerve inflammation does not initiate the degenerative phenomenon but represents a response to the denervation of muscles in ALS mice (Kano et al., 2012). Moreover, the observation of macrophages infiltration in the phrenic nerve concomitantly with minimal cervical MNs damage suggested a possible protective role of their phagocytic activity during nerve degeneration (Kano et al., 2012). Noteworthy, we have recently demonstrated that macrophages recruitment along motor axons and within the skeletal muscles correlates with a slower disease progression in mSOD1 mice (Nardo et al., 2016b; Vallarola et al., 2018). Intriguingly, in line with the putative protective role of infiltrated macrophages, it has been recently reported an association of PNS inflammation and longer disease duration in ALS patients (Schreiber et al., 2019).

1.3.2 ADAPTIVE IMMUNITY

The adaptive immune response has a longer duration, involves a complex orchestration of cellular and molecular responses, and is generated to a specific pathogen or antigen with a presence that may not have been resolved by the innate inflammatory response.

The main functions of the adaptive immune response are:

- Discrimination between self and non-self antigens;
- Generation of pathogen-specific effector pathways;
- Development of immunologic memory.

Cells associated with the adaptive immune response include tissue macrophages (e.g. microglia in the CNS) that can serve as antigen-presenting cells, B cells that mature into antibody-producing cells, and T cells with subsets that are critical to cellular immunity. The orchestrated immune response may further contribute to inflammation that can then enhance additional immune system involvement.

1.3.2.1 LYMPHOCYTES

In human, lymphocytes constitute ~20-40% of the total number of white blood cells. They are found in the circulation and are also concentrated in lymphoid organs and tissues (e.g. spleen, tonsils and lymph nodes) where the initial immune response is likely to occur. B and T lymphocytes develop

from hematopoietic stem cells (HSCs) that originate from bone marrow. HSCs then differentiate into multipotent progenitors (MPPs) which retain the potential to give rise to both myeloid and lymphoid lineage. The process of differentiation then proceeds to a common lymphoid progenitor (CLP), which can only differentiate into T, B or Natural Killer (NK) cells. Some lymphocytes migrate to the thymus, where they mature into T cells, others remain in the bone marrow, where they develop into B cells.

1.3.2.1.1 T LYMPHOCYTES

The maturation of T cells occurs in the thymus, where the CPL engraft. The cells arrived in the thymus are called double negative as they express neither the CD4 nor CD8 co-receptor. In the thymus these immature cells undergo three steps of selection: i) the T cell receptor beta (TCRβ)-selection, consisting in numerous rearrangements critical to creating a functional TCRβ chain to recognise antigens; ii) the positive selection, where the self-antigens are presented and iii) the negative selection, fundamental for the self-tolerance.

T cells are grouped in different subsets based on their function:

- $CD4^+$ T helper (Th) cells (Th1, Th2, Th17);
- $CD4^+$ T regulatory cells (T reg);
- $CD8^+$ cytotoxic T cells;
- Memory T cells.

Although the investigations on the involvement of inflammation in ALS pathogenic cascade have mostly focused on microglia and innate immunity, several studies reported T cells infiltration in *post mortem* material from ALS patients (Troost et al., 1990; Engelhardt et al., 1993).

Contradictory results were instead obtained from the characterisation of the circulating T cells population in ALS patients. Some studies reported an increased level of circulating $CD4^+$ T cells (Gustafson et al., 2017; Mantovani et al., 2009); conversely, others have found reduced numbers of T lymphocytes in the blood of ALS patients (Chen et al., 2014b). Similarly, $CD8^+$ cytotoxic T lymphocytes were found reduced (Mantovani et al., 2009), increased (Gustafson et al., 2017), or unchanged (Chen et al., 2014b) in ALS patients compared with healthy controls. These contradictory

results suggest that the different level of circulating T cells recorded in the different cohorts examined might be related to the variation in the immune responsiveness of individuals (McCombe et al., 2020).

In mSOD1 mice, $CD4^+$ T cells were observed in the lumbar spinal cord at the early phase of the disease, while both $CD4^+$ and $CD8^+$ populations were present at the end stage of the disease (Beers et al., 2008; Henkel et al., 2006; Chiu et al., 2008). Like microglia/macrophages phenotype, $CD4^+$ T cells are classified in two simplified classes: those that are neuroprotective (Th2 and T regulatory lymphocytes), and those that are pro-inflammatory/neurotoxic (Th1 and Th17 lymphocytes).

The role of T cells in ALS has been unravelled through the years. Several studies proposed a protective role of $CD4^+$ T helper lymphocytes. Indeed, it has been demonstrated that the crossbreeding of mSOD1 mice with mice lacking functional T cells ($RAG2^{-/-}$ mice) or depleted for $CD4^+$ cells worsened the disease progression increasing the production of pro-inflammatory cytokines (Beers et al., 2008). Similar results were obtained by the breeding of mSOD1 mice onto a TCRβ deficient background (Chiu et al., 2008). The protective role of $CD4^+$ T lymphocytes was further supported by the evidence that the reconstitution of the bone marrow or the passive transfer of *ex vivo* activated $CD4^+$ T cells improves neurological function and survival of ALS mice (Beers et al., 2008; Banerjee et al., 2008). Notably, it has been reported that the passive transfer of the T cell population enriched in T regulatory cells compared with whole $CD4^+$ T cell subset translated in a greater amelioration of the disease course in ALS mice, indicating a neuroprotective role of T reg lymphocytes (Beers et al., 2011a).

In-depth analysis in ALS models showed that the role (neuroprotective or neurotoxic) of $CD4^+$ T cells is strictly dependant from their interaction with microglia cells. In the early stage of the disease, Treg and Th2 cells predominate and release anti-inflammatory factors (e.g. IL4, IL10, TGFβ), which promote the M2 polarisation of microglia (Beers et al., 2011b; Zhao et al., 2004). In turn, M2 polarised microglia sustain and promote T reg and Th2 proliferation. In a synergic mechanism, the anti-inflammatory factors released by M2 microglia and Th2 and T reg lymphocytes inhibit the toxic function of Th1 cells (Beers et al., 2011b). As the disease progresses, a transformation occurs from

the supportive T reg/M2 response to the toxic Th1/M1 response. Following the release of inflammatory factors by the Th1 cells, microglia acquire a cytotoxic phenotype and, in a vicious circle, release toxic factors that foster the activity of Th1 lymphocytes. The toxic factors released by Th1 lymphocytes and M1 polarised astroglia (TNFα, IFNγ, IL6, IL1β) also induce the dysfunction of T reg cells allowing the progression and worsening of the disease symptoms (Zhao et al., 2013). According to the progressive T reg lymphocytes dysfunction, it has been found that the transplantation of T reg cells harvested from mSOD1 rodents at the stable phase, but not at the symptomatic stage of the disease, was sufficient to improve the clinical outcome of recipient double transgenic $RAG2^{-/-}/SOD1^{G93A}$ mice (Beers et al., 2011a).

Similar observations have been made in ALS patients. Indeed, a dysfunctional activity of T reg cells of ALS patients compared with healthy subjects (Beers et al., 2017), and an inverse correlation between circulating T reg cells level and the disease progression rate and severity have been reported (Beers et al., 2011a; Henkel et al., 2013; Sheean et al., 2018). This evidence suggests that both the reduced number and the impaired immunosuppressive function of T reg cells could influence ALS progression. Moreover, the protective role of T reg cells has been recently confirmed observing that their expansion by the peripheral injection of IL2 monoclonal antibody complexes reduced microglia activation, protected MNs and increased the life expectancy of mSOD1 mice (Sheean et al., 2018). Accordingly, the MIROCALS phase II clinical trial assessing the therapeutic potential of IL2 is currently ongoing (ClinicalTrial.gov NCT03039673).

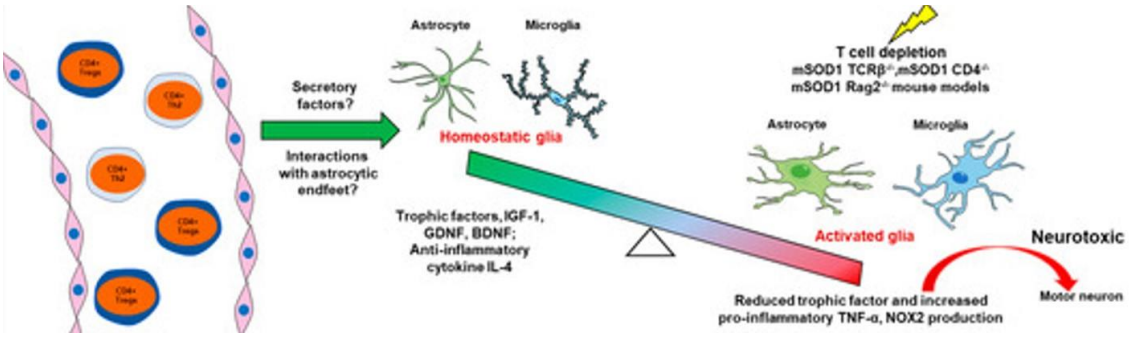

Figure 8: *$CD4^+$ T cells tip the balance between glial neurotrophism and neurotoxicity. Circulating $CD4^+$ T cells (specifically T reg and Th2 cells) via yet-to-be-identified mechanisms promote microglial and astrocyte production of trophic factors and anti-inflammatory cytokines. Depletion of $CD4^+$ T cells in mSOD1 mice via different genetic approaches switches them to an activated proinflammatory phenotype with neurotoxic properties (Modified from Iyer et al. 2018).*

Among the neurotoxic lymphocytes, also Th17 cells seem involved in ALS pathogenic cascade, although evidence regarding their involvement in the disease is narrow. Studies reported higher expression of Th17-related cytokines (i.e. IL17 and IL23) in biofluids and spinal cord of ALS patients (Saresella et al., 2013; Rentzos et al., 2010; Jin et al., 2020); however, little information was obtained in ALS models. Preclinical evidence showed a higher level and increased activation of Th17 cells upon facial nerve injury in mSOD1 compared to wild type mice, suggesting that the specific antigens released by damaged MNs primed the Th17 cells and promoted their recruitment into the CNS, where sustain the inflammation and neurodegeneration (Liu et al., 2017b; Ni et al., 2016).

Despite part of the innate immunity, Natural killer (NK) T lymphocytes are another T cell population involved in ALS. NK cells are pivotal in regulating the immune response since they release a plethora of pro- and anti-inflammatory factors, such as IL2, IL3, IL4, TNFα, IFNγ. NKT cells were found increased in peripheral blood of ALS patients and spinal cord, liver and spleen of mSOD1 mice (Rentzos et al., 2012; Finkelstein et al., 2011; Garofalo et al., 2020). Moreover, it has been reported that the treatment with an analogue of α-galactosylceramide, a compound that induced hypo-responsiveness of NK T cells, or the depletion of NK T subpopulation ameliorate the disease course and the histopathological features in ALS mice (Finkelstein et al. 2011; Garofalo et al. 2020). However, further investigations are needed to clarify the involvement of NK T lymphocytes in ALS.

In the last years, several studies have focused on $CD8^+$ T population of lymphocytes. Although preliminary evidence reported a delayed presence of $CD8^+$ T cells in the CNS of ALS mice (Beers et al., 2008), newly evidence described the infiltration of cytotoxic T lymphocytes as an early event in the disease (Nardo et al., 2018; Coque et al., 2019). $CD8^+$ T lymphocytes have been classically considered detrimental for MNs due to their antigen-specific effector cells capability. Indeed, $CD8^+$ T cells express the Fas ligand (FasL/CD95) (Peter et al., 2007), and it has been reported an increased susceptibility of MNs to Fas-mediated death (Raoul et al., 2002).

The role of $CD8^+$ T cells in ALS has been exploited through different experimental paradigms, such as crossbreeding mSOD1 mice with animals depleted (CD8a knock-out) (Coque et al., 2019) or defective (knock-out for the β2microglobulin subunit of MHCI) for $CD8^+$ T cells (Nardo et al., 2018)

or by the injection of anti-CD8a antibody (Komine et al., 2018). Results are controversial since CD8a$^{-/-}$/SOD1^{G93A} animals, or ALS mice injected with anti-CD8a antibody did not modify their survival while the β2microglobulin$^{-/-}$/SOD1^{G93A} mice showed and anticipation of the disease onset with prolonged survival.

Despite these discrepancies, CNS infiltrating CD8^{+} T cells release a high level of IFNγ compared to the wild-type circulating cytotoxic lymphocytes. This pro-inflammatory cytokine might contribute to the neurodegeneration promoting the somatic expression of MHCI by dying MNs (Coque et al., 2019). However, it is still unclear whether the elimination of damaged MNs is part of the cell death process or if dying neurons express autoantigens that trigger their removal through T cytotoxic lymphocytes. Accordingly, the production of IFNγ was reduced, and spinal MNs loss was delayed, in double transgenic β2microglobulin$^{-/-}$/SOD1^{G93A} mice. Nevertheless, the lack of CD8^{+} T cells severely affected the structure and function of peripheral motor axons anticipating the motor impairment in double transgenic β2microglobulin$^{-/-}$/SOD1^{G93A} mice (Nardo et al., 2018). This evidence suggests a pivotal role of CD8^{+} T cells in the immune-mediated axonal regenerative process occurring in the PNS. Suitably, we found a reduced infiltration of cytotoxic T cells within the sciatic nerve of the SOD1^{G93A} mice characterised by faster disease progression (Nardo et al., 2016b). These findings highlighted the complexity of ALS, in which the multifaceted activity of immune cells is affected by the disease progression and the environment with which they interact.

1.3.2.1.2 B LYMPHOCYTES

The maturation of HSC into B cells required various gene expression patterns and immunoglobulin heavy and light chain gene loci arrangements (Pelanda and Torres 2012). To ensure a proper development within the bone marrow, B cells undergo two types of selection: a positive selection, which occurs through antigen-independent signalling, and a negative selection, which occurs through the binding of self-antigen (LeBien and Tedder 2008). The achievement of the complete maturation takes place in the secondary lymphoid organs (e.g. spleen), where B cells became activated by binding the antigen via BCR (B cell receptor) (Yuseff et al., 2013).

The evidence concerning the involvement of B lymphocytes in ALS pathogenesis is narrow. Although no B cells infiltration has been observed in the spinal cord of human ALS (Troost et al., 1990; Engelhardt et al., 1993), increased concentrations of specific antibodies suggest an expansion of specific B-cell populations in patients. Indeed it has been reported increased immunoglobulin concentrations in peripheral blood of ALS patients compared with healthy subjects (Provinciali et al., 1988). Although with discordant results, some studies reported the presence of antibodies to ganglioside GM1 (Pestronk et al., 1989; Niebroj-Dobosz et al., 2009), calcium channel subunits (Smith et al., 1992), Fas (Sengun and Appel 2003) and lipoprotein-related protein 4 (Tzartos et al., 2014) in the sera of ALS patients. However, these autoantibodies have also been detected in others neurodegenerative disease and are not indicative for the specific MN degeneration (van den Berg et al., 1992; Bekircan-Kurt 2015; Shen et al., 2013). Therefore, the functional role of these autoantibodies in ALS is still speculative. However, they might be a driving factor in the disease progression of a limited population of ALS patients even if they do not contribute to the majority of cases (Lyon et al., 2019).

The contribution of B lymphocytes in ALS has also been addressed in the disease models, in which no B cells infiltration was recorded in the spinal cord (Chiu et al., 2008) albeit the production of autoantibodies has been confirmed. However, the B cells of mSOD1 mice did not exhibit an activated phenotype or increased responsiveness to pro-inflammatory stimulus compared with the wild type counterpart (Naor et al., 2009). Moreover, it has been demonstrated that the B cells depletion in $SOD1^{G93A}$ mice did not affect the disease course, suggesting their minor if not negligible contribution in the disease (Naor et al., 2009). Therefore, given the correlation with the severity of disease course, it is conceivable that autoantibody production is a secondary immunological consequence of neuronal death that may accelerate the neurodegenerative cascade (Niebroj-Dobosz et al., 2006). Although speculative, a better understanding of the role of autoantibodies and their ability to escape immune tolerance may pave the way for the development of disease-specific immunological signatures to be used for disease monitoring and to rate treatment response (Malaspina et al., 2015).

1.3.3 CYTOKINES

Cytokines are important signalling molecules synthesised by immune cells in peripheral tissues and blood, and by glial cells or non-resident immune cells in the CNS. One characteristic feature of cytokines is the functional redundancy and pleiotropism. Numerous cell types respond to cytokines, thereby regulating both homeostatic and pathological functions (Dinarello 2007).

It is well established that cytokines are mediators of both innate and adaptive immunity, therefore are involved in virtually every facet of immunity and inflammation, including antigen presentation, bone marrow differentiation, cellular recruitment and activation, and adhesion molecule expression (Borish and Steinke 2003). Once released, cytokines target the cells expressing the cognate receptors, which typically results in the recruitment of other immune cells and secretion of more cytokines.

In physiological condition, cytokines are expressed in low concentration in the CNS. However, in response to immune challenge, both cytokines and immune cells can pass through the BBB. Besides, non-neuronal cells (e.g. microglia, astrocytes) secrete cytokines on the brain side of the BBB inducing the neuroinflammatory phenomenon (Hanisch 2002).

The term cytokine encompasses a broad range of molecules which can be classified in chemokines, interferons, interleukins, lymphokines, tumour necrosis factor but generally not hormones or growth factors (despite some terminological overlap, e.g. TGFβ).

An extensive literature has been devoted to these molecules and their role in neurodegenerative diseases. Here, we will focus on the chemokine subclass of cytokines, in particular on the Monocyte Chemoattractant Protein 1 (MCP1), a.k.a C-C motif Chemokine Ligand 2 (CCL2).

1.3.3.1 CHEMOKINE

Chemokines – chemotactic cytokines – are the largest family of cytokines in humans. Their name derives from "*-kinos*," which is Greek for "movement". Indeed, chemokines play a vital role in cell migration through venules from the blood into tissue and viceversa, and in the induction of cell movement in response to a chemical (chemotactic) gradient by a process known as chemotaxis (Baggiolini 1998).

Inducible chemokine expression is generally modulated by pro-inflammatory stimuli to promote the chemotaxis of macrophages, neutrophils, and other lymphocytes to sites of injury, infection or phlogosis. However, chemokines are also involved in leucocyte degranulation (Mackay 2001), hematopoiesis (Youn et al., 2000), and angiogenesis (Belperio et al., 2000). Accordingly, they can be grouped in: inflammatory chemokines, that control leucocytes recruitment into the inflamed/damaged tissue, and homeostatic chemokines, that fulfil housekeeping functions (Zlotnik and Yoshie 2000).

Chemokines are small proteins of approximately 80 amino acids in size that are classified into main subfamilies based on the sequential positioning of the first two of four highly conserved cysteine residues: CXC (α subfamily), CC (β subfamily) and CX3C (δ subfamily) (Zlotnik and Yoshie 2000). In the largest α and β subfamilies the first two cysteines are adjacent (CC motif) or separated by one amino acid residue (CXC motif) respectively. In contrast, in the δ subfamily chemokines have three amino acids between the first two cysteine residues (CX3C motif) (Zlotnik and Yoshie 2000). The CXC chemokines can be further divided into two subgroups, 'ELR' and 'non-ELR,' based on the presence/absence of the Glu-Leu-Arg motif before the first cysteine. An exception is represented by the γ subfamily, in which only one N-terminal cysteine residue is present (XC motif) (Kelner et al., 1994).

Even though the sequence identity between chemokines varies from about 20% to 90%, their sequences overall are highly conserved. Moreover, chemokines acquire substantially the same fold. These structures consist of a flexible N-terminus and N-terminal loop, followed by a three-stranded antiparallel β-sheet onto which is folded a C-terminal α-helix. The highly conserved cysteine residues interact to form disulphide bridges that are crucial at maintaining the structural integrity of the protein, which is a prerequisite for chemokine binding to their respective receptors (Campbell 2003; Miller and Mayo 2017).

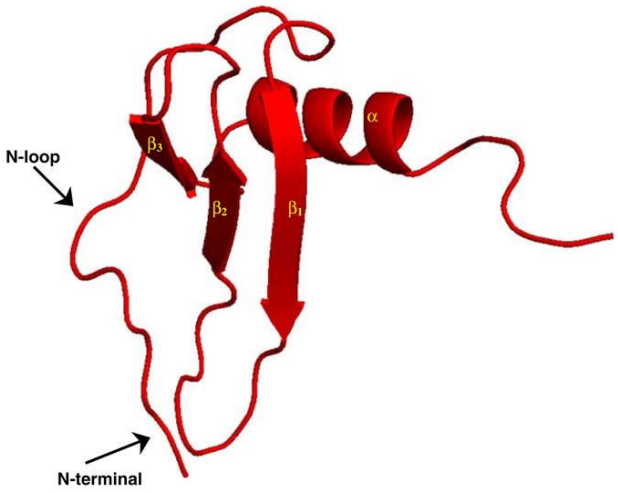

Figure 9: *Schematic representation of the three dimensional chemokine structure* (Rajagopalan and Rajarathnam 2006).

Chemokines exert their function by interacting with two classes of receptors: conventional chemokine receptors (cCKRs) and atypical chemokine receptors (aCKRs). Chemokine-bound cCKRs typically transduce signals through pertussis toxin-sensitive Gαi G-proteins, β-arrestins and JAK-STAT pathways, ultimately leading to cell migration, adhesion and/or a variety of other biological responses (Bachelerie et al., 2014; Kehrl 2006). Chemokines are thought to initially tether to their cognate cCKR via the extracellular loops and N-terminus of the receptor. Once a chemokine is tethered to a cCKR, its unstructured N-terminus enters the heptahelical bundle of the receptor to induce a conformational change that is translated into intracellular signals (Kufareva et al., 2015). This classical two-site model of chemokine-receptor interaction is probably oversimplistic. Indeed, recent studies suggested that the two supposedly independent ligand-binding sites can be physically and allosterically linked and that additional interactions between chemokine and receptor are likely to be involved in ensuring full receptor activation (Kleist et al., 2016).

Atypical chemokine receptors are structurally related to cCKRs but do not couple to many, if any, of the signal transduction pathways activated by the conventional receptors. This may be in part due to the absence, or modification, of appropriate signalling motifs on the intracellular surface of aCKRs, such as the canonical DRY (Asp–Arg–Tyr) motif (Nibbs and Graham 2013). aCKRs structurally resemble cCKRs but cannot directly initiate migratory responses. Instead, they scavenge, sequester or transport chemokines to control cCKR-driven responses, and can also regulate co-expressed cCKRs (Nibbs and Graham 2013). However, chemokine scavenging is not restricted to aCKRs;

indeed, the activation of cCKR is accompanied by the internalisation of chemokine-cCKR complexes (Volpe et al., 2012).

1.3.3.1.1 MONOCYTE CHEMOATTRACTANT PROTEIN 1 (MCP1 / CCL2)

Monocyte chemoattractant protein 1 (MCP1), a.k.a. C-C Motif chemokine Ligand 2 (CCL2), is a member of the β chemokine subfamily. This family encompasses several small, secreted, chemotactic cytokines, named after their best-known function of attracting cells (Baggiolini 1998). MCP1 is the first discovered human CC chemokine. Located on chromosome 17 (chr.17, q11.2), human MCP1 is composed of 76 amino acids and is 13kDa in size (Van Coillie et al., 1999). MCP1 belongs to a family consisting of other three members: MCP2/CCL8, MCP3/CCL7 and MCP4/CCL13. In mouse, four proteins have been identified (MCP1, MCP2, MCP3 and MCP5/CCL12) that share substantial amino acid identity to the human's chemokines. However, the cross-species assignments of orthologs among these genes are not entirely clear. The murine MCP1, MCP2 and MCP3 are orthologs of the human MCP1, MCP2 and MCP3 proteins. However, no mouse ortholog has been described for human MCP4 and, viceversa, no ortholog in the human genome was found for the murine MCP5 (Van Coillie et al., 1999).

Human MCP1 is produced as a precursor molecule containing a hydrophobic N-terminal signal sequence of 23 amino acids. After cleavage of the signal peptide portion, a mature protein of 74-76 amino acids is secreted. Different molecular mass forms of MCP1 have been purified. Still, these seem to be caused by post-translational modifications (e.g. O-glycosylation) (Jiang et al., 1990), which have been shown to slightly reducing its chemotactic potency (Proost et al., 1998).

Mutation studies identified the regions 10-13 and 34-35 as critical for the biological activity of MCP1 (Beall et al., 1996). Deletion of residues at the N-terminus, which is crucial for the receptor recognition and signalling, resulted in the loss of chemokine activity. Conversely, the modification of the C-terminus does not affect the chemotactic capability of MCP1 (Proost et al., 1998). However, some of these mutant forms of MCP1 act as chemokine antagonists (Gong and Clark-Lewis 1995).

MCP1 is expressed by a variety of cells, such as endothelial cells, smooth muscle cells, fibroblasts, epithelial cells, astroglia, T cells (Cushing et al., 1990; Strieter et al., 1989; Standiford et al., 1991;

Barna et al., 1994; Hayashi et al., 1995; Owen et al., 2007). However, myeloid cells (i.e. monocytes/macrophages) are the primary source of the chemokine (Rollins 1997).

MCP1 expression is inducible, triggered upon exposure to inflammatory stimuli, such as LPS, interleukins (IL1, IL4, IL6), TNFα, TGFβ, IFNγ, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), M-CSF and GM-CSF (Van Coillieet al., 1999; Kumar and Boss 2000; Luther and Cyster 2001; Choi et al., 2017; Yoshimura 2018). MCP1 is usually released to exert a potent chemotactic activity by binding the C-C chemokine receptor type 2 (CCR2), which is expressed by several cell subsets, among these monocytes (Han et al., 1998), macrophages (Gendelman et al., 2009), B and T lymphocytes (Carr et al., 1994; Frade et al., 1997; Allavena et al., 1994), dendritic cells (Zhu et al., 2000), neutrophils (Johnston et al., 1999), but also by endothelial cells (Weber et al., 1999) and smooth muscle cells (Hayes et al., 1998). CCR2 is a cCKR (seven transmembrane G protein coupled receptor, GPCR) which can also be activated by non-selective ligands including MCP3 and MCP4 (Gouwy et al., 2004; Wain et al., 2002). However, among these ligands, MCP1 is the most potent inducer of the signal transduction pathways leading to monocytes transmigration (Sozzani et al., 1994).

MCP1-CCR2 binding activates several intracellular cascades mediated by numerous interactors, such as JAK2/STAT3, MAP kinases, phosphatidylinositol 3-kinases (PI3K), which are involved in cells migration (Wainnet al., 2002; Mellado et al., 1998; Kuang et al., 1996), and phospholipase C, which promotes calcium release (Kuang et al., 1996). Two alternatively spliced forms of CCR2 have been discovered (CCR2A and CCR2B) which differ only in their C-terminal tails (Charo and Taubman 2004) and possibly, in the downstream signalling (Sanders et al. 2000).

Besides, MCP1 can bind two atypical chemokine receptors (no GPCR), aCKR1 and aCKR2, which show broad specificity for inflammatory chemokines. aCKR1 is expressed by red blood cells and blood vessels endothelial cells (but not leucocytes) and participates at regulating the chemokine abundance (scavenging activity) and transcytosis (Nibbs and Graham 2013). Lymphatic endothelial cells and mouse-innate like B cells are the unique cells expressing aCKR2 (Nibbs et al., 2001; Hansell et al., 2011).

Notably, CCR2 is considered the key regulator of Ly6c^{high} monocytes infiltration within inflamed tissues and mobilisation from the bone marrow (BM) under steady-state condition (Tsou et al., 2007). However, the BM mobilisation of monocytes is also governed by aCKR2, which is further involved in controlling their abundance in the circulation (Savino et al., 2012).

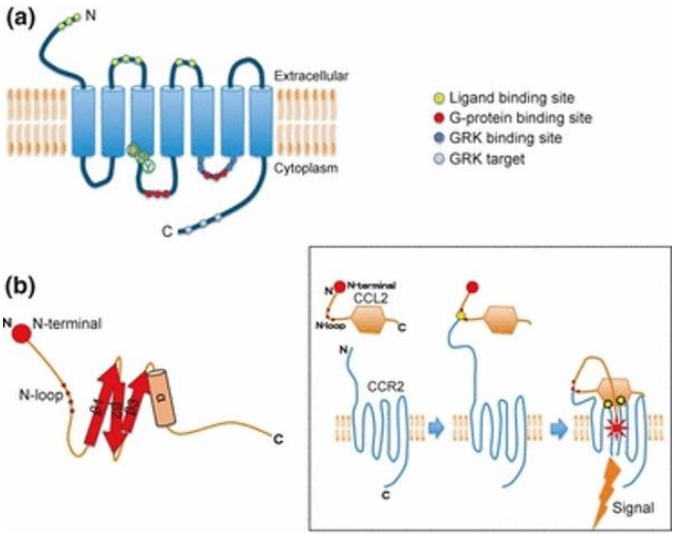

Figure 10: *(a) Schematic diagram of the CCR2 protein: it consists of four extracellular domains, seven transmembrane domains, and four intracellular domains. The DRY (Asp–Arg–Tyr) motif, which is important for G-protein-mediated signal transduction, is located in the juxtamembrane region of the second intracellular loop. G-protein binding sites are also located on the second and third intracellular loops. G-Protein receptor kinase (GRK) binds to the third intracellular loop and phosphorylates the GRK targets on the C-tail to initiate internalisation of the CCR2 receptor after ligand binding. (b) Schema of MCP1/CCL2 and receptor binding. MCP1 consists of a short N-terminal region and an extended N-loop region, followed by three β-strands and an α-helix. High-affinity binding of each ligand occurs between the N-loop of the chemokine and the N-terminal of the receptor, followed by interactions between the body of MCP1 and the extracellular loops of CCR2. The N-terminal segment of MCP1 binds within the receptor and initiates intracellular signalling (inset)* (Yamasaki et al. 2012).

Intriguingly, MCP1 is not merely a guidance cue during immune cells extravasation toward the site of phlogosis as it also controls the monocyte adhesion to vascular endothelium promoting the expression of two members of the β2 family of integrins, CD11b and CD11c (Vaddi and Newton 1994; Jiang et al., 1992). Moreover, it has been proposed the involvement of MCP1 in the macrophage polarization and subsequent cytokines release. However, the comprehension of the biological function of the chemokine remains elusive. Numerous evidence showed that the direct *in vitro* stimulation with the chemokine favoured the M1 polarization of macrophages promoting the release of pro-inflammatory factors (TNFα, IL1β, IL6) (Wang et al., 2014b; Sodhi and Biswas 2002). Coherently, MCP1-null mice displayed an M2 phenotype characterised by the increased expression of TGFβ and Arginase 1 (Nio et al., 2012). In contrast, it has been reported that the

stimulation of human myeloid cells with MCP1 led to an increase of $CD14^+/CD206^+$ cells (i.e. M2 macrophages) (Roca et al., 2009) and that CCR2-null mice exhibited a reduced number of M2 polarised peritoneal macrophages compared to controls (Sierra-Filardi et al., 2014).

These pieces of evidence showed that the effect of MCP1 might be dependent on the intrinsic activation and polarisation state of the monocytes at the time of stimulation, suggesting that the MCP1-mediated polarisation fingerprint may be strictly dependant from the inflammatory context.

Role of MCP1 in the Central Nervous System

The role of the MCP1/CCR2 axis in the CNS is still controversial.

Increased expression of MCP1 is usually associated with the neuroinflammatory phenomenon (Conductier et al., 2010; Sawyer et al., 2014; Semple et al., 2010b). Accordingly, MCP1 upregulation has been identified in several neurodegenerative/neuroinflammatory disorders such as ALS (Wilms et al., 2003; Baron et al., 2005; Nagata et al., 2007), Parkinson disease (Reale et al., 2009), Alzheimer disease (Ishizuka et al., 1997) and in their respective rodent models (Janelsins et al., 2005; Kalkonde et al., 2007; Henkel et al., 2006).

Typically, MCP1 is secreted by activated astrocytes to attract microglia cells to the site of neuronal injury, where they phagocyte the pathogens and cellular debris (Ransohoff et al., 1993; Hurwitz et al., 1995; Glabinski et al., 1996). Moreover, it has been reported that also microglia, neurons and endothelial cells are a source of MCP1 during pathological conditions (Berman et al., 1996; Thibeault et al., 2001; Howe et al., 2017). Notably, the CNS-recruited monocytes trough MCP1 chemotactic guidance are in turn a source of the chemokine, implying the presence of autocrine regulation that perpetuates cells recruitment and activation during phlogosis (Calvo et al., 1996; Gunn et al., 1997; Gourmala et al., 1997).

Intriguingly, it has been observed that the brain microvasculature endothelial cells respond to MCP1 enhancing the permeability of the BBB via RhoA signalling and cytoskeleton reorganization (Stamatovic 2003; 2005). These effects were attenuated in MCP1-treated animals that had previously been depleted of peripheral macrophages, indicating a direct impact of this chemokine on the endothelial cells of the BBB and an indirect effect involving leucocytes recruitment and

subsequent changes in the BBB permeability (Stamatovic et al., 2006; Song and Pachter 2004). According to the neurotoxic role of MCP1/CCR2 axis, the depletion of chemokine or its receptor reduced the inflammatory phenomenon in several rodent models of neuroinflammatory diseases (Zhang et al., 2018a; Varvel et al., 2016; Dimitrijevic et al., 2007). Specularly, the chemokine overexpression exacerbated the pathological features (Chen et al., 2003; Joly-Amado et al., 2020; Yamamoto et al., 2005).

However, considerable evidence reported the physiological expression of the chemokine in microglia cells, astrocytes and neurons (Goazigo et al., 2013; Banisadr et al., 2005). Besides, a distinct pattern of MCP1 and CCR2 expression had been identified at different embryonic stages, implying a role for this pathway during brain development (Meng et al., 1999; Rezaie et al., 2002). Neural progenitors cells are attracted in an MCP1-mediated manner to the site of injury, where promote regeneration (Belmadani et al., 2006). Moreover, the genetic ablation of CCR2 or MCP1 in a mouse model of Alzheimer disease enhanced the accumulation of Amyloid β (Aβ) and accelerated the cognitive decline in a manner that correlated with the gene dosage (El Khoury et al., 2007; Kiyota et al., 2013) suggesting an initial protective role of the chemokine in stimulating the phagocytic activity of microglia cells.

Interestingly, it has been reported that the *in vitro* stimulation of microglia with MCP1 did not induce morphological changes nor the release of neurotoxic factors, suggesting that other stimuli might be necessary to drive the modifications that led to the acquirement of toxic function when the chemokine levels are elevated (Hinojosa et al., 2011). Moreover, it has been observed that the *in vitro* stimulation of the astrocytic CCR2 enhanced their survival, through the activation of NFκB and Akt signalling pathways, promoted the release of neurotrophic factors, and reduced the production of neurotoxic molecules even after a pro-inflammatory stimulus (Quinones et al., 2008; Kalehua et al., 2004; Semple et al., 2010a). Besides, it has been proven that the MCP1 released from astrocyte exerted a neuroprotective role in several *in vitro* neurotoxic paradigms such as excessive glutamate exposure (i.e. excitotoxicity), oxygen-glucose deprivation (Rosito et al., 2012) or methyl mercury administration (Godefroy et al., 2012). The chemokine release by astrocytes is pivotal for

the maintenance of the homeostasis within the CNS. Indeed, it has been recently reported that astrocytes derived from a mouse model of Spinal Muscle Atrophy (SMA) showed a decreased production of MCP1 that translated in reduced support and axonal elongation of SMA or wild type isolated MNs. Notably, this deficit could be restored through the exogenous administration of the chemokine (Martin et al., 2017).

Notably, the axonal outgrowth seems directly regulated by neuronal MCP1 as demonstrated by the reduced motility and axonal elongation observed in the NSC34 motor neuron-like cells expressing a splicing variant of Survival Motor Neuron protein (axonal SMN) upon chemokine knocking-down *in vitro* (Locatelli et al., 2012). Intriguingly, the therapeutic action of the mesenchymal stem cells in rodent models of spinal cord injury (SCI) is strictly mediated by the release of MCP1. Indeed, it has been reported that the chemokine exerts a neuroprotective effect by fostering the polarisation of the recruited macrophages towards the M2 pro-healing phenotype, reducing the neurons susceptibility to the excitotoxic phenomenon and also directly promoting the neurite arborisation (Papa et al., 2018; Matsubara et al., 2015). Corroborative evidence demonstrated that the preconditioning peripheral nerve injury in the SCI animal model increased the production of MCP1 by neurons promoting the dorsal root ganglia outgrowth through the direct M2 polarisation of the recruited macrophages (Kwon et al., 2015). Coherently, the overexpression of the chemokine through the intrathecal injection of an AAV5_MCP1 engineered viral vector led to the increased of the neurite outgrowth (Niemi et al., 2016).

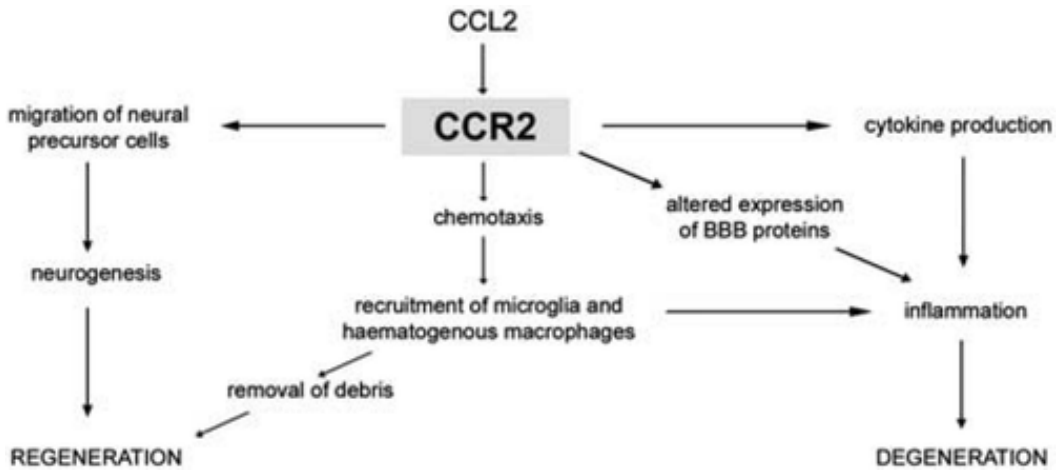

Figure 11: *The roles of CCL2(MCP1)/CCR2 axis during neuroinflammation. MCP1 induces the recruitment of macrophages, production of cytokines, and direct alteration of the expression of endothelial cell tight-junction proteins to increase blood-brain barrier permeability, which contributes to inflammation, potentially exacerbating neuronal loss. MCP1-mediated*

macrophage accumulation may also be beneficial, as these phagocytic cells remove myelin debris, which otherwise inhibits regeneration. Furthermore, MCP1 is chemotactic for neural precursor cells and thus, may influence repair after injury by enhancing neurogenesis. (Semple et al., 2010b).

In the ALS context, MCP1 is classically associated with the neuroinflammatory phenomenon. Indeed, high levels of the chemokine have been found in serum and particularly in the CSF of ALS patients (Wilms 2003; Simpson et al., 2004; Henkel et al., 2004; Baron et al., 2005; Tanaka et al., 2006). Notably, it has also been reported a direct correlation between the MCP1 levels in the CSF and the severity or the speed of the disease progression in patients (Tanaka et al., 2006). However, this evidence was disproved by more recent clinical examinations (Martínez et al., 2020). Nevertheless, the elevated concentration of the chemokine in the CSF surmises an intrathecal production rather than a CNS diffusion from blood circulation. Indeed, MCP1 immunoreactivity was detected within the microvasculature, astroglia cells, MNs and infiltrating macrophages in the lumbar spinal cord of ALS patients (Henkel et al., 2004; Baron et al., 2005).

Although ALS mice are more amenable to study specific pathways, the involvement of MCP1-CCR2 axis in the disease is far to be elucidated. It has been recently reported that the antibody-mediated neutralisation of the chemokine reduced the immune cells infiltration in the CNS and ameliorated the disease course in mSOD1 mice (Garofalo et al., 2020). Moreover, similarly to patients, it has been shown a gradual upregulation of MCP1 transcript in the spinal cord of mSOD1 mice as the disease progresses, and the increased expression of the chemokine by activated microglia and MNs of ALS mice compared with wild type animals (Henkel et al., 2006). Notably, this study reported an upregulation of the chemokine already in 15 days old mSOD1 mice, i.e. before the microglia cells activation and the massive production of pro-inflammatory cytokines underlying the establishment of the neuroinflammatory event. As suggested in a mouse model of Alzheimer disease (Kiyota et al., 2013), this evidence might imply a protective action on this signalling in the early disease stage that might evolve in neurotoxic during the ALS progression (Henkel et al., 2006). Indeed, it has been reported that, at the disease onset, microglia strongly upregulate MCP1 and others chemokines fostering the recruitment of monocytes within the CNS (Butovsky et al., 2012). Moreover, an *in vitro*

study showed that mSOD1 microglia cells exhibit pro-inflammatory fingerprints which include the increased secretion of MCP1 upon LPS stimulus (Sargsyan et al., 2009).

Even more controversial is the evidence concerning the CCR2 expression in the CNS of ALS mice. The initial difficulty stands in the characterisation of the cellular subtype expressing the chemokine receptor. Constitutive CCR2 expression has been reported in neurons, astrocytes and microvascular endothelial cells of wild type mice (Banisadr et al. ,2002; Ge et al., 2008). However, this evidence was not corroborated by the characterisation of transgenic mice in which the receptor sequence was substituted by the red fluorescent reporter gene (CCR2-RFP mice), in which a specific CCR2 expression was recorded in the leucocytic population (e.g. monocytes/macrophages and T cells) (Saederup et al., 2010). A further explorative study in ALS mice identified the receptor exclusively on the membrane of activated astrocytes (Kawaguchi-Niida et al., 2013). Conversely, it has been recently reported the CCR2 expression by microglia, neurons and infiltrating monocytes, but not astrocytes, of mSOD1 mice (Komiya et al., 2020).

Given the opposite results and the poor aptitude of mSOD1 at recruiting $CCR2^{+}$ immune cells in the CNS (Chiu et al., 2009; Kunis et al., 2015), we still lack a definitive picture illustrating both the expression pattern and the role of MCP1-CCR2 axis in the CNS pathology of ALS.

Role of MCP1 in the Peripheral Nervous System

CNS axons do not spontaneously regenerate after injury in adult mammals. In contrast, PNS axons readily regenerate, allowing the recovery of function after peripheral nerve damage. Therefore, understanding factors underlying the PNS regeneration or its inhibition is essential for developing therapies for individuals with axonopathies, including ALS (Moloney et al., 2014).

After damage, peripheral axons degenerate and regrow following a process termed “Wallerian degeneration”, so named in honour of the clinician Augustus Volney Waller who described it in 1850. Notably, the typical ovoidal structures of the Wallerian degeneration have been observed within the degenerating motor axons of ALS patients and models (Tian et al., 2016).

Successful axon regeneration relies on a robust regenerative response of injured axons and the coordinate contribution of non-neuronal cells, including immune cells (Gaudet et al., 2011). Indeed,

considerable evidence reported that innate (monocytes/macrophages, neutrophils) and adaptive immune cells (T lymphocytes) are massively involved in PNS degeneration and regeneration (Gaudet et al., 2011; Benowitz and Popovich 2011; Chen et al., 2015b; Bombeiro et al., 2016). The immune response is fundamental to turn the peripheral nerve tissue into an environment permissive to regeneration by removing inhibitory signals (e.g. myelin and cellular debris) and by upregulating neurotrophic features. The characteristics of an efficient immune response are the rapid onset and conclusion, and the fine and orchestrated interplay between the involved interactors (resident or infiltrating cells) and the molecules that they release (Rotshenker 2011).

Upon nerve damage, several cytokines involved in inflammation, immune response and chemotaxis are upregulated, including IL1β, TNFα, IFNγ, RANTES (CCL5), MIP1α (CCL3) and MCP1 (CCL2) (Perrin et al., 2005; Taskinen and Roytta 2000; Kleinschnitz et al., 2005). Experimental evidence demonstrated that almost all the resident (Schwann cells, endoneurial macrophages) or infiltrating (monocytes-derived macrophages, T lymphocytes) cells involved in response to nerve degeneration are a source of cytokines, among these MCP1. Intriguingly, also the degenerated motor axons increased the expression of MCP1 to mediate the interaction with the infiltrating macrophages (Kwon et al., 2015). These pieces of evidence highlight the relevance of this signalling pathway in the PNS degeneration and regeneration mechanisms (Stratton et al., 2020; Tofaris et al., 2002).

Schwann cells (SCs) are the first line response upon peripheral nerve damage and are the primary mediators in triggering many of the events in Wallerian degeneration (Jessen et al., 2015). In the absence of the physical contact to the axon, and following the stimulation by the Toll-like Receptor ligands released by the damaged axons, SCs de-differentiate to an immature non-myelinating (ensheathing) phenotype (Gaudet et al., 2011). De-differentiated SCs upregulate MAC2 protein to acquire a phagocytic phenotype (Reichert et al., 1994) fundamental to remove the myelin debris that contains molecules, such as MAG (myelin-associated glycoprotein) and OMgp (oligodendrocyte-myelin glycoprotein), that inhibit the axonal regrowth (Huang et al., 2005). Once de-differentiated, SCs downregulate miR-327 allowing the release MCP1 (Zhao et al., 2017b) to recruit hematogenous monocytes within the injury site (Taskinen and Roytta 2000; Tofaris et al.,

2002; Subang and Richardson 2001), a process also favoured by the breakdown of the blood nerve barrier (BNB). Together, the endoneurial activated macrophages release cytokines and chemokine, such as LIF, TNFα and MCP1 to amplify the recruitment of monocytes from circulation (Mueller et al., 2003) and VEGF to alter the permeability of the BNB microvessels (Shimizu et al., 2011).

This evidence demonstrates the pivotal role of macrophages and the signalling pathway that govern their infiltration upon nerve damage. Indeed, in the later stage of the peripheral nerve degeneration, monocytes-derived macrophages are the major cells contributing to remove myelin and axonal debris and thus to create a favourable milieu to attempt to regenerate (Barrette et al., 2008; Chen et al., 2015b).

The pivotal role of MCP1-CCR2 axis and macrophages recruitment in peripheral nerve response to injury has been demonstrated through several experimental approaches. Indeed, the impairment of the regenerative capacity of the injured nerve during ageing is strictly related to the downregulated expression of MCP1 by macrophages of old mice but not to their migratory activity compared to macrophages derived from young animals (Stratton et al., 2020). Moreover, studies performed in the mice lesioned sciatic nerve showed that the *in situ* administration of MCP1-neutralizing antibody suppresses the macrophage-mediated response and significantly impairs the myelin clearance within the damaged nerve (Perrin et al., 2005). Similarly, the administration of antibodies to CCR2 decreased the circulating monocytes level also impeding their infiltration in the injured sciatic nerve, thus hampering the nerve regeneration (Lindborg et al., 2017). Corroborating evidence showed that mice genetically depleted for the chemokine or its receptor exhibited a reduced macrophages accumulation in the distal sciatic nerve and dorsal root ganglia (DRG) after nerve injury (Niemi et al., 2013; Siebert et al., 2000; Lindborg et al., 2017). Similarly, the depletion of monocytes/macrophages through the administration of macrophages deactivators, such as minocycline or clodronate liposome (Keilhoff et al., 2007; Chen et al. 2015b), or by using CD11b conditional knock out mice (CD11b-TK$^{mt\text{-}30}$ mice) (Barrette et al., 2008), decreased the recruitment of these immune cells in the distal stump of the nerve severely affecting the axonal regeneration and the locomotor function of injured animals. Notably, the injection of MCP1 within the DRG

increased macrophages recruitment and, differently from other chemokines (i.e. CX3CL1 and CCL3), promoted the neurite outgrowth instructing the infiltrating macrophages toward the M2 anti-inflammatory phenotype (Kwon et al., 2015). This observation suggested that the participation of the MCP1-recruited macrophages in the axonal regeneration is not limited to their phagocytic activity toward the cellular debris but are actively involved in the releasing of trophic factors. Indeed, it has been demonstrated that upon injury both M1 and M2-polarised macrophages are recruited (Tomlinson et al., 2018) and that the ratio of pro-healing to pro-inflammatory population, rather than the extent of macrophage presence within the damaged nerve, regulates the regenerative outcomes (Mokarram et al., 2012). However, the exact mechanisms governing macrophage polarization in peripheral nerve injury models are still poorly understood (Zhang et al. 2019). Pro-healing macrophages release some trophic factors, such as VEGF, that is essential for the formation of new blood vessels and Schwann cells guidance (Cattin et al., 2015). Accordingly, the angiogenesis mediated by macrophages in the acellular nerve allograft promotes the efficient SCs and T cells repopulation finally leading to nerve regeneration and functional recovery (Pan et al., 2020). Moreover, macrophages directly govern the mature or immature status of SCs, thus promoting or inhibiting the remyelination upon injury (Stratton et al., 2018).

Although ALS is considered a distal axonopathy (Moloney et al., 2014), the PNS degeneration and regeneration and the stream of these mechanisms have been so far underestimated. Indeed, the macrophages and immune response involvement in the early pathology of the peripheral compartment has not been investigated in ALS. However, studies in SOD1^{G93A} mice showed the presence of peripheral macrophages along degenerating nerve fibres in the ventral root, sciatic nerve and intramuscular axons (Chiu et al., 2009). Accordingly, upregulation of MCP1 and CD68 transcripts were recorded in the sciatic nerve of mSOD1 mice suggesting that peripheral nerve inflammation is probably not the cause of the degeneration, but rather a response to the damage (Kano et al., 2012; Deng et al., 2018). Moreover, although it has been reported the BNB leakage in mSOD1 mice, the recruitment of macrophages is reduced compared to non-transgenic littermates upon a nerve crush. This observation suggests that a deficit/inhibition of the immune response may

occur in ALS mice that might be responsible for the impaired nerve regeneration following an injury (Deng et al., 2018).

Although MCP1 preferentially recruits monocytes (Rollins 1997), CCR2 is also expressed by activated T lymphocytes (Bonecchi et al., 1998; Luther and Cyster 2001). Notably, it has been demonstrated that the inflammatory factors released by adaptive immune cells potentiate the phagocytic activity of macrophages, indicating a pivotal role of T lymphocytes in the regenerative mechanisms upon a nerve injury (Bombeiro et al., 2016). Accordingly, it has been recently observed a reduced innate and adaptive immune cells infiltration within the sciatic nerve of fast progressing $SOD1^{G93A}$ mice that correlated with reduced expression of MCP1 along motor axons compared with slow progressing ALS mice (Nardo et al., 2016b). Suitably, the absence of cytotoxic T lymphocytes in double transgenic $SOD1^{G93A}$/β2microglobulin$^{-/-}$ animals severely affected the peripheral axon structure resulting in anticipation of the motor deficit (Nardo et al., 2018).

The recent characterization of the PNS of ALS patients corroborated the preclinical evidence. Indeed, it has been reported a direct correlation between the PNS inflammation and longer disease duration (Schreiber et al., 2019). Moreover, the gene expression profile of motor nerves of ALS patients revealed the downregulation of CCR2, suggesting that a defective immune cells infiltration at the site of degeneration may be implicated in ALS pathology (Riva et al., 2016).

Role of MCP1 in the skeletal muscle

Skeletal muscle is one of the most abundant tissues in the human body. It accounts for ~45% of the total body mass and is necessary for generating forces for movement.

Progressive muscle loss can result from mechanical traumas, metabolic disorders, inherited genetic diseases (e.g. ALS, Duchenne muscular dystrophy, Charcot-Marie-Tooth disease) (Pansarasa et al., 2014; Shin et al., 2013; Jani-Acsadi et al., 2015) or can also be a consequence of peripheral nerve injuries, chronic kidney disease, diabetes, and heart failure (Kalyani et al., 2014).

Up to a certain threshold (~20%), skeletal muscle has the capability of regenerating the lost tissue thanks to its high adaptability and healing potential (Tedesco et al. ,2010). Beyond this threshold, the remaining muscular tissue is unable to regenerate its function fully. This loss of skeletal muscle

with lasting functional impairment, defined as “volumetric muscle loss” (Grogan and Hsu 2011), can substantially impact the quality of life of patients by significantly reducing the functionality of the locomotion system.

Several stages compose the process of muscle regeneration upon injury: i) necrosis of the injured muscle cell; ii) activation, proliferation and differentiation of muscle stem cells (satellite cells); iii) maturation of the newly formed muscle fibres and, finally, iv) the remodelling of muscle fibres. Acute inflammation and immune cells play critical roles in almost all stages of muscle regeneration (Yang and Hu 2018).

Previous studies have suggested that chemokines are important actors in the regeneration of the skeletal muscle (Nicholas et al., 2015; Warren et al., 2005; 2004). Indeed, increased expression levels of several chemokine ligands and their cognate receptors have been found in muscle biopsies of patients and animal models of muscular dystrophy or inflammatory myopathies (Confalonieri et al., 2000; De Paepe and De Bleecker 2013; Porter et al., 2003). Among the cytokines upregulated upon a muscle injury (e.g. TNF family, interleukins, interferons, α and β chemokines), MCP1 seems pivotal in triggering the regenerative process of skeletal muscle (Lu et al., 2011a). Indeed it has been reported that myogenic precursor cells (a.k.a. satellite cells), injured muscle fibres, epimysium and perimysium resident macrophages and recruited monocytes are source of MCP1 (Chazaud et al., 2003; Brigitte et al., 2010; Lu et al., 2011a). Notably, it has been observed a significant upregulation of MCP1 transcript 4-8 hours following muscle crush in mice, preceding chemokine expression and the resulting infiltration of macrophages in the injured muscle (Nicholas et al., 2015). This evidence highlights the pivotal role of MCP1 in orchestrating the immune-mediated response upon muscle injury.

Several preclinical studies showed that following muscle injury (acute trauma, toxins administration, exercise or diseases, etc.) the damaged tissue initiates a stereotypical inflammatory response in which the number of intramuscular leucocytes rapidly increase (Yang and Hu 2018; Chazaud 2020; Tidball 2017; Rigamonti et al., 2014; Pizza 2008). Resident macrophages are pivotal in sensing the damage occurred and, once activated, secrete chemokines, such as neutrophil

chemoattractant CXC chemokine ligand 1 (CXCL1) and MCP1, to promote the recruitment of neutrophils and monocytes respectively (Tidball 2005; Brigitte et al., 2010). Within hours, neutrophils invade damaged muscle and reach maximum numbers at approximately 12-24 hours post-injury, after which they rapidly return to near-normal numbers (Pizza 2008). Neutrophils stimulate host defence through the phagocytosis of cellular debris and releasing ROS and proteases (Tidball 2011). Besides, neutrophils also sustain and amplify the inflammatory process releasing cytokines, such as MIP1α and MCP1, thus attracting circulating monocytes within the damaged tissue (Scapini et al., 2000). Intriguingly, muscle resident T lymphocytes exert unexpected, essential roles at initiating the cascade of events leading to wound healing. Indeed, it has been reported that CD8α deficiency led to a reduction in the release of MCP1, that translates in the impairment of macrophages recruitment and thus muscle regeneration upon cardiotoxin muscular injection in mice (Zhang et al., 2014a).

The high chemotactic MCP1 gradient established within the damaged muscle promotes the massive recruitment of Ly6c^{high} monocytes, which extravasate and enter in a muscular environment that is enriched with pro-inflammatory cytokines, such as IFNγ and TNFα (Warren et al., 2002; Collins and Grounds 2001; Cheng et al., 2008). Therefore, monocytes-derived macrophages are initially polarised toward the M1 phenotype and secrete pro-inflammatory and chemotactic factors (e.g. IFNγ, IL1β, TNFα and MCP1) and ROS to facilitate the removal of cellular debris and the recruitment of immune cells to the damaged area, therefore amplifying the ongoing inflammatory response (Dort et al., 2019; Tidball et al., 2014; Villalta et al., 2009). Preclinical studies showed that M1 polarised macrophages reach peak number 24-48 hours after the acute injury, after that they switch toward the pro-healing anti-inflammatory M2 phenotype (Arnold et al. ,2007; Varga et al., 2016). M2 polarised macrophages are actively involved in promoting the resolution of the inflammatory process by releasing a wide array of anti-inflammatory factors (IL4, IL13, Arginase 1), pro-resolving lipids (15Δ-PGJ2) and trophic factors (IGF1) (Dort et al., 2019; Arnold et al., 2007; Chazaud 2020).

However, as previously discussed, the M1/M2 signature represents an oversimplification of the inflammatory milieu within the degenerating/regenerating muscles. Although a mixture of M1 and

M2 polarized macrophages have been observed following a muscle injury (Heredia et al., 2013; Wang et al., 2018;), their phenotypic switch and temporal and spatial recruitment represent a critical step to accomplish the proper muscle regeneration. Indeed, it has been reported that macrophages interact with satellite cells to regulate myogenesis (Chazaud et al., 2009; Madaro et al., 2019). The soluble factors released by pro-inflammatory macrophages stimulate myogenic precursor cells proliferation, while anti-inflammatory cytokines from M2 macrophages promote their differentiation (Arnold et al., 2007; Tidball et al., 2014; Varga et al., 2016). Similarly, the macrophage skewing also regulates the balance between muscle fibrosis and tissue remodelling, directly inhibiting (M1 macrophages) or promoting (M2 macrophages) fibroadipogenic progenitors expansion (Lemos et al., 2015).

According to the classic kinetic of immune cells infiltration, T lymphocytes are the last cells entering in the damaged muscle peaking around 3-5 days post-injury (Fu et al., 2015; Tidball 2017). Although the presence of T cells was initially identified as a pathologic phenomenon of muscle damage (Orimo et al., 1991; McLennan 1996), the loss or gain of $CD8^+$ or $CD4^+$ T lymphocytes affected and rescued muscle regeneration capacity, respectively (Fu et al., 2015; Zhang et al. 2014a). Similarly, the loss of T reg cells, whom infiltration coincides and sustains the phenotypic M1->M2 transition of macrophages, impaired muscle repair and regeneration upon damage (Burzyn et al., 2013; Castiglioni et al., 2015; Kuswanto et al., 2016).

Altogether these observations indicate that muscle regeneration is a collection of highly synchronised processes involving several cellular, molecular and signalling responses in which the coordinate effort of inflammation and regeneration is fundamental for the achievement of an efficient repair program following an injury (Bentzinger et al., 2013). Particularly, the intramuscular inflammatory signalling plays a critical role in mediating the regenerative response of the injured muscle and must be finely regulated. This because the inflammatory cytokine expression is capable of promoting both muscle growth and muscle loss (Muñoz-Cánoves et al., 2013; Howard et al., 2020).

In vivo studies in CD11b-diphtheria toxin receptor (CD11b-DTR) transgenic mice (Wang et al., 2014a; Arnold et al., 2007) or following the administration of clodronate liposome or Etoposide (Liu et al., 2017b; Xiao, Liu and Chen 2016; Bryer et al., 2008; Dumont and Frenette 2010) have unequivocally demonstrated that the depletion of macrophages severely impairs skeletal muscle regeneration. Accordingly, the role of MCP1-CCR2 axis has been extensively investigated.

Several studies identified MCP1-CCR2 signalling pathway as the primary entry route of the Ly6c^{high} monocytes into the injured muscle (Saclier et al., 2013a; 2013b; Shireman et al., 2007; Lu et al., 2011a). Suitably, studies performed in MCP1 deficient mice showed a reduced macrophages recruitment that translated to a poor muscle regeneration (Shireman et al., 2007; Lu et al., 2011a; Martinez et al., 2010). Intriguingly, intravenously injected MCP1-deficient bone marrow monocytes could not enter in wild-type injured muscle despite the chemotactic gradient established within the damaged tissue upon barium chloride injection (Lu et al., 2011a). This evidence suggests that MCP1 expressed by circulating monocytes may exert an autocrine function fundamental for their transmigration toward the damaged site and that chemokine expression by bone marrow-derived cells and injured muscle is required for proper muscle regeneration (Lu et al., 2011a). The same results have been obtained in CCR2 knock-out mice, in which the impaired egression of monocytes from the bone marrow resulted in poor muscle regeneration (Contreras-Shannon et al., 2007; Ochoa et al., 2007; Lu et al., 2011b; Sun et al., 2009; Warren et al., 2005). The deficient macrophages recruitment in CCR2 knockout mice led to the increase of pro-inflammatory (TNFα, MIP1β, MCP1, MCP3, MCP5), pro-angiogenic (KC-GRO, IL3 and GM-CSF) and pro-hematopoietic (SCF and SDF1) cytokines release depicting a scenario similar to the so-called “inflammaging”, condition well known to impair the tissue regeneration (Melton et al., 2016). This dysregulation was rebalanced by wild type bone marrow-derived cells, which restored the physiological inflammation in CCR2 deficient mice, and partially in MCP1 depleted animals, re-establishing the inflammatory response within the injured muscle (Sun et al., 2009; Lu et al., 2011a; Melton et al., 2016). Notably, the regenerative impairment recorded in MCP1 deficient mice was not severe as observed in CCR2 depleted mice,

suggesting a compensatory action of other chemokines (e.g. CCL5) in the absence of the CCR2 specific ligand (Martinez et al., 2010; Lu et al., 2011a).

Besides MCP1-CCR2 axis, other chemokines signalling pathways participate in the muscular response to damage. Indeed, preclinical studies have shown that the depletion of CXCL16 (Zhang et al., 2009) or the fractalkine receptor (CX3CR1) (Zhao et al., 2016) severely impaired the muscle regeneration reducing the macrophages recruitment and their phagocytic capability respectively. Conversely, the administration of CXCL12 in rat crushed muscles activated and mobilised the $CD34^{+}/CXCR4^{+}$ precursor cells residing in the bone marrow or blood circulation, thus improving the muscle regeneration (Brzoska et al., 2012). Noteworthy, these studies reported a direct action of the chemokines, including MCP1 (Warren et al., 2005), on the muscular progenitor cells.

This evidence highlighted the pivotal role of chemokine in muscle degeneration and regeneration upon an acute injury, suggesting a dual mechanism of action. On one hand, through the establishment of a chemotactic gradient within the injured tissue, chemokine recruit leucocytes which, in turn, release cytokines (e.g. TNFα) and trophic growth factors (e.g. IGF1) that promote the activation and commitment of satellite cells (Tidball, Dorshkind, and Wehling-Henricks 2014). On the other hand, chemokines directly interact with the myogenic precursor cells influencing their response following an injury (Warren et al., 2005; Zhang et al., 2009; Brzoska et al., 2012).

The involvement of MCP1 signalling in the degenerating muscle has not been investigated in ALS. However, it has been reported a progressive increase of the chemokine transcript in the skeletal muscle of $SOD1^{G93A}$ mice at the later stage of the disease (Manzano et al., 2011). Nevertheless, the contribution of immune cells, particularly macrophages, in the degenerative or regenerative mechanism of the skeletal muscle during the disease course has attracted considerable attention in the last years. Indeed, T cells and macrophages infiltrate, and a significant increase of CD68 and CD45 transcripts have been observed in skeletal muscle biopsies of ALS patients compared with healthy subjects (Jensen et al., 2016). This evidence confirmed the previous observations, indicating a close association between the infiltration of the immune and the extent of muscle fibres destruction in ALS (Troost et al., 1992). Similarly, it has been reported a progressive upregulation

of CD11b and CD68 markers and macrophages infiltration in the skeletal muscle of rodent models of the disease (Chiu et al., 2009; Van Dyke et al., 2016; Wang et al., 2017; Trias et al., 2017). Intriguingly, the studies performed in $SOD1^{G93A}$ mice demonstrated that the more severely affected muscles, such as tibialis anterior, showed greater macrophages infiltration compared with the diaphragm (Chiu et al., 2009). However, the role of macrophages in ALS degenerating muscle is still unclear. Some preclinical studies demonstrated that the reduction of the macrophages recruitment was sufficient to preserve the neuromuscular junctions from denervation and thus ameliorate the motor deficit in ALS models (Van Dyke et al., 2016; Trias et al., 2017; Wang et al., 2017). Conversely, it has been reported that the reduced macrophages infiltration in the hind limb skeletal muscles was associated with an earlier onset and a more aggressive disease course in $SOD1^{G93A}$ mice (Vallarola et al., 2018). Noteworthy, it has been suggested that ALS macrophages possess a dysregulated capability, as demonstrated by the promoted skeletal muscle regeneration observed in mSOD1 mice following whole wild type bone marrow transplantation (Corti et al., 2004).

These pieces of evidence highlighted the complexity and pleiotropy of the immune response, depicting it as a real-time example of an evolving system. Mainly, in the ALS field, the observations hitherto collected paint a picture of a rising systemic immune response as the disease progresses. However, whether the immune changes are causative and therefore represent an attractive therapeutic target, or whether they are a secondary downstream effect of the dysfunctions occurred in the CNS is still debated. Furthermore, the recent evidence suggested a dual role of the immune response in the CNS compared with the peripheral compartment (Chiu et al., 2009; Dibaj et al., 2011) making the comprehension of the immune mechanism involved in ALS even more challenging.

OVERALL OBJECTIVES and SPECIFIC AIMS

Chapter II

OVERALL OBJECTIVES and SPECIFIC AIMS

Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disease characterised by a higher heterogeneity in term of clinical manifestation and speed of disease progression (Ticozzi and Silani 2018; Bendotti et al., 2020). Arguably, such heterogeneity stems from the different mechanisms involved in its pathogenesis. Indeed, the knowledge so far acquired through the study of ALS mouse models showed that multiple mechanisms contribute to motor neuron (MN) injury (Mejzini et al., 2019). Moreover, it has become clear that ALS is a non-cell autonomous disease with other cell types within the central nervous system (CNS) actively contributing to the disease including microglia, astrocytes and immune cells (Chiot et al., 2019; Thonhoff, Simpson, and Appel 2018).

However, the impressive amount of knowledge acquired did not yield the expected outcomes in term of therapeutic benefit. Several bodies of evidence demonstrated that the preservation of MNs is per se not sufficient to tangibly counteract the disease (Rouaux et al., 2007; Gould et al., 2006). Accordingly, it has been observed that skeletal muscle atrophy and axonal degeneration are early events in the disease pathogenic cascade, anticipating MN loss and symptoms manifestation (Clark et al., 2016; Azzouz et al., 1997). In agreement with this observation, ALS has been recently described as a distal axonopathy whereby skeletal muscles actively contributes to a retrograde signalling cascade that culminates with MN death (Moloney et al., 2014; Dadon-Nachum at al., 2011).

Mounting experimental evidence highlights the different contribution of the inflammatory response in the CNS compared with the periphery (i.e. nerves and muscles) (Dibaj et al., 2011; Chiu et al., 2009). Indeed, while the aberrant glial cells activation, T cells infiltration and the resulting release of pro-inflammatory factors drive neurodegeneration, the successful axon and muscle regeneration depends on the coordinated efforts of immune cells which, besides removing cellular debris, release factors that support wound healing (Deng et al. ,2018; Gaudet et al., 2011; Sass et al., 2018; Van Dyke et al., 2016).

It is, therefore, possible to postulate that the immune response can actively influence the progression of the disease, promoting phenomena of neuroprotection and/or neurotoxicity (Lyon

et al., 2019; Wosiski-Kuhn et al., 2019). To shed light on the nature and temporal development of immune response in central and peripheral compartments affected by the disease could be a useful tool to discover new biomarkers and identify targets for the development of precise therapeutic strategies aimed to slow down ALS progression.

The assumption of a pivotal role of the peripheral immune response in governing the speed of the disease progression has been recently validated in our laboratory following the characterisation of two mouse strains (C57 and 129Sv) carrying the same copies of human mutant SOD1 transgene ($SOD1^{G93A}$) but exhibiting remarkable differences in term of disease onset, progression and overall survival (Marino et al., 2015; Nardo et al., 2016a). Our studies revealed that, despite the two ALS models exhibiting the same extent of MNs loss during the disease progression (Marino et al., 2015), the fast progressing mice (129Sv $SOD1^{G93A}$) showed earlier muscle denervation that correlates with a reduced macrophages infiltration within the peripheral compartment compared with the slow progressing ALS mice (C57 $SOD1^{G93A}$) (Nardo et al., 2016b; Vallarola et al., 2018). Further analyses showed a strong downregulation of one of the most potent chemotactic agent for haematogenous monocytes, such as Monocyte Chemoattractant Protein 1 (MCP1), a.k.a C-C Motif Chemokine Ligand 2 (CCL2), in fast progressing compared with slow progressing ALS mice at both central and peripheral level (Nardo et al., 2013, 2016b).

The evidence hitherto collected suggests that the immune system might be pivotal in delaying muscular denervation and triggering the regeneration of the neuromuscular system, thus regulating the speed of disease progression of the two ALS models. At the same time, MCP1 seems to fulfil a critical role in these processes. Indeed, the involvement of MCP1-mediated pathway in nerve and muscle regeneration has been recently suggested (Niemi et al., 2016; Shireman et al., 2007) along with its engagement also as a neuroprotective factor (Locatelli et al., 2012; Papa et al., 2018).

To verify the heftiness of these observations and to clarify whether a proinflammatory chemokine might exert a protective role in ALS, this project will aim to characterise the effect of the early

induction of MCP1 on the clinical outcomes and histopathological/biomolecular features of fast and slow progressing ALS mice.

SPECIFIC AIMS

To induce the chemokine an innovative approach is chosen consisting in the single injection of a self-complementary Adeno-Associated Virus serotype 9 (scAAV9) engineered with the sequence encoding for the murine *MCP1* gene (scAAV9_MCP1).

The characterisation of the effect of scAAV9_MCP1 injection will be achieved through the following steps:

- ✓ In the first section of this Thesis, the assessment of the monocytes/macrophages recruitment and their inflammatory fingerprint within the skeletal muscles of fast and slow progressing ALS patients will be performed to corroborate the relevance of the preliminary preclinical observations.
- ✓ The second section of this project will be devoted to the characterisation of the best route of administration of the scAAV9 to target the entire motor unit (i.e. MN soma, axons and skeletal muscle) in ALS mice. A comparison between the intracerebroventricular (i.c.v) and intramuscular (i.m.) administration of the scAAV9 engineered with the Green Fluorescent Protein (GFP) reporter gene sequence will be tested to reach the target avoiding the secondary side effects following the systemic induction of a pro-inflammatory chemokine.
- ✓ The third section of the project will aim to understand whether an early (pre-symptomatic disease stage) induction of MCP1 might ameliorate the motor ability and symptoms progression of fast and slow progressing ALS mice. An in-depth characterisation of the effect of chemokine induction on the lower motor units will be performed in the two $SOD1^{G93A}$ models focusing at the symptomatic stage of the disease.
- ✓ In the last section, we will examine the early regenerative mechanisms activated by slow progressing C57 $SOD1^{G93A}$ mice six weeks after the MCP1 induction. We will also analyse whether the preservation of the upper motor units is pivotal in slowing down the disease progression of ALS mice.

MATERIALS and METHODS

Chapter III

3.1 MURINE MODELS

In this study, female transgenic SOD1^{G93A} mice on C57BL/6J (C57^{G93A}) and 129S2/Sv (129Sv^{G93A}) genetic background, and their corresponding non-transgenic (Ntg) littermates were used. Both SOD1^{G93A} mouse strains were maintained on the homogenous background for more than 15 generations.

Mice were housed 4/5 per standard cages in specific pathogen-free and controlled environmental condition (temperature: 21±1°C; relative humidity: 60% and 12 hours of light). All the experimental procedures were conducted in conformity with institutional guidelines that comply with national (D.L. n.26, G.U. 4 March 2014) and international guidelines and laws (EEC Council Directive 86/609, OJ L 358, 1, 12 December 1987, Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All the experimental procedures were reviewed and approved by the intramural ethical committee and the Italian Ministry of Health.

3.1.1 SOD1^{G93A} ALS MOUSE MODELS

The ALS animal models used in the experiments are transgenic mice expressing ~20 copies of the gene encoding for the human Superoxide Dismutase 1 (SOD1) enzyme with the point mutation Glycine to Alanine in position 93 (SOD1^{G93A}). All the lines are hemizygous for the transgene (Gurney et al., 1994), so SOD1^{G93A} male mice were repeatedly backcrossed with non-transgenic (Ntg) female mice.

The animals used in the experiments are on two different homogeneous genetic backgrounds:

- C57BL/6J-SOD1^{G93A} (C57 SOD1^{G93A}) are derived from B6.Cg-Tg(SOD1*G93A)1Gur/J (Jackson Laboratories) and crossed with non-transgenic C57BL/6J female mice.
- 129S2/SvHsd-SOD1^{G93A} (129Sv SOD1^{G93A}) have been generated in the laboratory by repeated backcross of C57 SOD1^{G93A} males with 129Sv Ntg female mice, obtaining transgenic mice on the homogeneous 129Sv genetic background.

3.1.2 MICE GENOTYPING

Genotyping was performed on tail biopsies collected from mice at weaning age (~21 days), to identify transgenic ($SOD1^{G93A}$) and non-transgenic (Ntg) animals. Samples were wholly digested by overnight incubation at 55°C in Direct- PCR Lysis Buffer (Viagen Biotech, Los Angeles, California, USA) containing 0.1 µg/µl of Proteinase K (Promega). The following day, after the inactivation of the Proteinase K at 85°C for 30 minutes, the Polymerase Chain Reaction (PCR) was performed using the Life Express Cycler TC-96/G/H(b)A (Bioer Technology Co. Ltd, Hangzhou, Zhejiang, China). 50 ng of extracted DNA for each animal was used as a substrate for qualitative PCR in a mix containing 1X PCR Buffer, Go-Taq DNA Polymerase (0.25U), deoxyNTPs (250µM each), specific forward (5'->3', CATCAGCCCTAATCCATCTGA) and reverse (5'->3', CGCGACTAACAATCAAAGTGA) primers (0.5µM each) in a final volume of 10 µl. All reagents were purchased by Promega, except for primers that were synthesised by Invitrogen.

Sequences, annealing temperatures and PCR programme for hSOD1 primers are reported in the table below.

PCR programme:		
Single denaturation cycle	94°C for 2 minutes	
Denaturation Annealing Elongation	94°C for 45 seconds 58°C for 45 seconds 72°C for 60 seconds	x 30 times
Final elongation step	72°C for 10 minutes	

Table 8: *PCR assay programme for the detection of the human SOD1 (hSOD1) transgene.*

Amplicons were resolved in 1% agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium) 1:30000 in Tris-Acetate-EDTA (40mM Tris, 0.35% vol/vol acid acetic, 1 mM EDTA) and visualised under UV using ChemiDoc XRS system (BioRad).

3.2. CLINICAL DISEASE PROGRESSION IN $SOD1^{G93A}$ MICE

3.2.1 BEHAVIOURAL ANALYSIS

The onset and the progression of the disease have been monitored through behavioural analysis as previously described (Pizzasegola et al., 2009). Briefly, mice were trained per two weeks to perform

the Paw Grip Endurance (PaGE) test starting at the pre-symptomatic disease stage (i.e. 6 weeks of age). After the two weeks of training, body weight was recorded, and mice were tested for the motor performance twice a week by a blinded operator.

3.2.1.1 BODY WEIGHT MONITORING

The weight of mice was measured before every behavioural test session. The weight loss was calculated by subtracting the maximum weight value from each registered weight.

3.2.1.2 PAW GRIP ENDURANCE TEST

The grip strength test is a simple non-invasive method designed to evaluate mouse muscle force *in vivo*, by taking advantage of the animal tendency to grasp a horizontal metal bar or a grid with its paws. Mice were placed on a horizontal metallic grid, which was then gently inverted. The latency to fall of each mouse was recorded. The test was considered passed if the mouse was able to cling to the grid for 90 seconds. In case of failure, the measurement was repeated three times, and the best performance of the session was considered for the statistical analysis.

The test has been performed on C57SOD1^{G93A} mouse model and the latency was evaluated. Concerning 129svSOD1^{G93A} mouse model, the performance obtained in the grip strength test was assessed through a score, which is calculated as indicated by Lauranzano *et al.* (2015):

$$score = Ttot - \sum_{i=1}^{n} \frac{Tdouble\ i}{2} + \sum_{j=1}^{n} \frac{Tsingle\ j}{4}$$

Briefly, the Ttot is the time spent hanging before falling from the grid, n is the number of events in which both the hind limbs (i) or the fore/hind limb paw (j) were detached from the grid, the Tdouble i is the number of seconds the i-th event lasted, the Tsingle j is the number of seconds the j-th event lasted. The paw detachment was considered significant above 3 seconds.

3.2.2 DETERMINATION OF THE PATHOLOGICAL STAGE

The clinical onset was determined by the inability of the mouse to reach the maximum score at the PaGE test in two consecutive sessions of the behavioural analysis. The time occurred before the disease onset was considered as the pre-symptomatic stage of the disease.

3.3 MICE TREATMENT

Fast and slow progressing mice were treated through the injection of a self-complementary adeno-associated virus serotype 9 (scAAV9) engineered with the enhanced green fluorescent protein (*eGFP*) or *MCP1* murine gene under cytomegalovirus (CMV) promoter. An empty vector was used as control.

3.3.1 scAAV9 VECTOR ENGINEERING

The scAAV9 engineered with the *eGFP* or *MCP1* gene sequence and the empty vector (control) were produced by Virovek (Hayward, CA, USA) following the steps below:

Steps	Description
1	Cloning gene of interest (GOI) into Virovek's AAV shuttle plasmid
2	Generation of Bacmid and purification of Bacmid DNA
3	Transfection of Sf9 cells to generate baculovirus
4	Amplification of baculovirus and titration
5	Production of AAV and CsCl purification
6	Desalting, filter sterilization, and AAV titration

Figure 12: *Description of AAV production by Virovek (www.virovek.com/company/aav-production-technology).*

The scAAV9 vector was chosen considering the great tropism of the serotype 9 toward skeletal muscles. Moreover, it has been demonstrated the capability of the scAAV9 in mediating a widespread gene delivery from muscles to the spinal cord (Benkhelifa-Ziyyat et al., 2013).

3.3.2 TREATMENT WITH THE ENGINEERED scAAV9

Intra-cerebro-ventricular (i.c.v.) injection of scAAV9

The i.c.v. injection of scAAV9_eGFP was performed in P1 (postnatal day 1) mice as previously described (Glascock et al., 2011; Gholizadeh et al., 2013). Briefly, mice underwent a single bilateral i.c.v. injection of $4.48x10^{12}$ vg/µL of scAAV9_eGFP opportunely diluted in 0.1% w/v trypan blue in sterile phosphate buffered saline (PBS) (2µl/ventricle). Cryo-anesthetised P1 mice were placed on a fibre-optic light to illuminate the midline and transverse sutures, which were used as a guide for the identification of neonates cerebral ventricle, and injected with a sterilised glass micropipette attached to a 3mL Hamilton syringe (Hamilton, Reno, NV) through a polyethene catheter. The sterile glass micropipette was inserted 2mm deep, perpendicular to the skull surface, at a location

approximately 0.25mm lateral to the sagittal suture and 0.50–0.75mm rostral to the neonatal coronary suture and left in place for 30 seconds after discontinuation of plunger movement to prevent backflow. After the surgical procedure, mice were placed under an infra-red lamp to restore the physiological body temperature and brought back into the cage with the dam once the normal movement and general responsiveness were reinstated.

Intramuscular (i.m.) injection of scAAV9

In general, the AAV vector is considered less immunogenic in rodents. However, the immune response against the AAV vector and transgene product is the main obstacle spotted in large animals and humans (Qiao et al., 2011). To avoid the establishment of an illicit immune response, for the i.m. treatment, the viral vector dose was maintained in the range from $5x10^8$ to $5x10^{10}$ vg/site, as recommended by Gruntman *et al.* (2013).

Adult mice underwent a single bilateral i.m. injection of $2{,}18x10^{10}$ vg/µL of the engineered (*eGFP* or *MCP1* gene) scAAV9 or the empty vector as control. The injection has been performed on both hind limb (*Tibialis Anterior*, TA; *Gastrocnemius Caput Medialis*, GCM; *Gluteus Maximus*, GM) and forelimb (*Triceps Brachii*, TB) muscles following the protocol previously described by Gruntman *et al.* (2013) to allow the targeting of both cervical and lumbar motor neurons (Tosolini et al., 2013; Mohan et al., 2014). Briefly, mice were anaesthetised with isoflurane inhalation, fur was shaved, and the limbs were taped in position to visualise the target muscles. A 30-gauge needle was inserted into the muscle mid-belly, to facilitate the targeting of neuro-muscular junction (Tosolini et al., 2013; Mohan et al., 2014), and 10µL of the engineered or empty vector opportunely diluted in sterile PBS was injected in each muscle. The syringe needle was left in place for 30 seconds after discontinuation of plunger movement to prevent backflow.

After the surgical procedure, mice were placed under an infrared lamp to restore the physiological body temperature and brought back into their cages once the normal movement and general responsiveness were reinstated. To allow the full recovery from the anaesthesia, animals were tested in the behavioural analysis two days after the i.m. injection.

3.4 HUMAN SAMPLES

The skeletal muscle biopsies were selected from the Telethon Neuromuscular Bank of Tissues and DNA samples and kindly provided by Dr Pegoraro and Dr Soraù (University of Padua).

Biopsies were collected from n=20 ALS patients with spinal onset. The categorisation in fast and slow progressing patients was performed according to the calculated progression rate (ΔFS) as previously indicated by Kimura *et al.* (2006).

$$\Delta FS = \frac{48 - \text{ALSFRS-R at "time of diagnosis"}}{\text{duration from onset to diagnosis (month)}}$$

After the collection, bioptic samples were frozen into the liquid phase of cooled isopentane for no more than 45 seconds and finally stored at -80°C until use.

ID	Gender	ALS type	Onset	Diagnosis	ALSFRS-R score at the diagnosis	Biopsy site	Biopsy collection	ΔFS
9957	M	sALS	Jan 2010	Nov 2015	37	LVL	Mar 2015	0.16
10275	M	sALS	Jan 2014	Feb 2017	42	LVL	Dec 2016	0.16
9837	M	fALS	Apr 2013	Mar 2014	46	LVL	July 2014	0.18
10008	M	ALS	Jan 2013	Sept 2015	43	LVL	July 2015	0.18
10064	F	sALS	Feb 2013	Oct 2015	42	LVL	Oct 2015	0.19
10429	M	sALS	Jan 2014	Feb 2018	38	LVL	Dec 2017	0.20
9785	M	sALS	Apr 2013	June 2014	45	LVL	Apr 2014	0.21
9992	M	ALS	Dec 2012	Jan 2016	36	LVL	May 2015	0.24
10039	M	sALS	Sept 2014	Aug 2015	43	LVL	Aug 2015	0.45
10430	F	sALS	May 2017	May 2018	40	LVL	Jan 2018	0.67

9887	M	fALS	Feb 2014	Dec 2014	41	LVL	Oct 2014	0.70
9899	F	sALS	Mar 2014	Jan 2015	41	LVL	Nov 2014	0.70
10347	M	ALS	Oct 2016	Apr 2017	43	LVL	May 2017	0.83
10425	F	sALS	Mar 2017	Nov 2017	41	LVL	Dec 2017	0.88
9926	M	sALS	Jan 2012	Nov 2014	17	LVL	Feb 2015	0.91
10337	M	sALS	July 2016	Oct 2017	34	LVL	May 2017	0.93
10518	M	sALS	Jan 2018	Oct 2018	38	LVL	Sept 2018	1.11
9984	M	sALS	Jan 2014	May 2015	27	LVL	May 2015	1.31
10358	M	ALS	Sept 2017	Dec 2017	44	LVL	June 2017	1.33
9865	F	sALS	Mar 2013	Nov 2014	33	RQF	Sept 2014	1.88

Table 9: *List and features of ALS patients whose muscles have been analysed in this study (LVL, left vastus lateralis muscle; RQF, right quadriceps femoris muscle).*

3.5 HISTOLOGICAL ANALYSIS

3.5.1 TISSUE COLLECTION

Mice were anaesthetised with a mix of ketamine (1.75 mg/Kg) and medetomidine (1 mg/Kg) and transcardially perfused with 50 ml of 0.1M PBS pH 7.4. Following the blood removal, the skeletal muscles (TA, GCM, GM and TB) were dissected out and immediately frozen in cooled isopentane. At the same time, the vertebral column was post-fixed overnight in a solution of 4% paraformaldehyde in 0.1M PBS. The following day the vertebrae were removed, and the spinal cord was transferred to 30% sucrose solution in 0.1M PBS and conserved at 4°C for at least two O/N (overnights). Before the use, the spinal cord was divided in the cervical, thoracic and lumbar segments, which were individually embedded in Tissue-Tek OCT compound (Sakura,

Zoeterwounde, The Netherlands), and finally frozen in cooled n-pentane. All tissues collected were stored at -80°C until required.

Sections 30µm thick were obtained by cutting the spinal cord in the coronal plane on a cryostat at -20°C (CM1950, Leica Biosystems). The L2-L5 lumbar and C2-C7 cervical level of the spinal cord were chosen for the experiments. Serial longitudinal (20 µm) or coronal (12 µm) cryosections of the TA and TB muscles were collected on poly-lysine objective slides (VWR International).

3.5.2 INDIRECT IMMUNOFLUORESCENCE

Free-floating sections of spinal cord or glass adhered sections of the TA and TB muscles were treated for 1h with a blocking solution composed of NGS and Triton X-100/Tween20 at the appropriate concentration in 0.01M PBS. Subsequently, the sections were incubated overnight at 4°C with the primary antibody diluted in PBS containing NGS and Triton X-100. Then, after three washes in PBS, samples were treated for 1h with the appropriate secondary antibody conjugated to fluorochromes with various wavelengths (Alexa Fluor 488, 594 or 647; Molecular Probes, Invitrogen), diluted 1:500 in PBS added with NGS. The following markers represent an exception: DAPI (4',6-diamidino-2-phenylindole), Neurotrace (recognise the Nissl substance present in the neurons perikaryon) and Wheat Germ Agglutinin (WGA) lectin, since they are directly conjugated with the fluorophore. After three washes, sections were mounted on slides and cover-slipped with Fluorsave (Calbiochem, Nottingham, UK). Controls sections were incubated without the primary antibody. The antibodies used in this study are listed below.

Antibody	Host species	Dilution	Supplier
Choline Acetyltransferase (ChAT)	goat	1:200	Merck Millipore
Glial Fibrillary Acidic Protein (GFAP)	mouse	1:2˙500	Merck Millipore
Green fluorescent protein (GFP)	chicken	1:750	Merck Millipore
Macrosialin (CD68)	rat	1:200	Biorad
Myogenic determination gene (MyoD)	rabbit	1:100	DSHB
Neurofilament heavy polypeptide (NF200)	rabbit	1:1˙000	Abcam
Neutrophil Elastase	rabbit	1:300	Abcam
Paired box 7 (Pax7)	mouse	1:400	DSHB

Table 10: *List of the antibodies used for the immunohistochemistry analysis.*

3.5.3 SUCCINATE DEHYDROGENASE (SDH) STAINING

For the muscle fibre composition, serial transverse cryosections (10 µm) from the mid-belly region of the TA muscle were air-dried and then incubated at 37°C for 30' in phosphate buffer (0.2M, pH 7.6) containing 13.5mg/mL Na-succinate (Sigma-Aldrich, St. Louis, MO, USA) and 0.5mg/mL of nitro blue tetrazolium (Sigma-Aldrich, 0.29mg/mL of buffer solution). Sections were finally fixed with 4% paraformaldehyde, dehydrated in a graded series of ethanol (70%, 90% and 100%) for 5' each and dipped in xylene. Finally, the objective slide was cover-slipped with DPX mounting medium (Sigma Aldrich).

3.5.4 IMAGE ANALYSIS

For motor neurons count analysis, a total of 12 serial ChAT-stained sections were analysed with an IX81 microscope equipped with a confocal scan unit FV500 with three laser lines: Argon-Krypton (488 nm), Helium-Neon red (646 nm), and Helium-Neon green (532 nm) and a UV diode (Olympus, Tokyo, Japan) using a 10× objective. The neuron areas were analysed with Fiji software (Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA). Only neuronal somas with an area ≥ 400 μm^2 were considered for quantitative analysis of MN numbers (Friese et al., 2009).

Fluorescence-labelled sections images (3/5 per animal) of the TA and TB muscle were analysed with an Olympus virtual slide system VS110 (Olympus, Center Valley, USA) and acquired at 20x magnification.

The images of SDH stained muscles were acquired with a CCD colour camera (Color View III; Soft Imaging System, GmbH), using AnaliSYS software (Soft Imaging Systems, GmbH, ver. 3.2) at 20x magnification.

For cells counting analyses (macrophages density, satellite cells and centralised myonuclei) and the SDH staining, a systematic random sampling procedure was applied as previously described (Nardo et al. 2018; Geuna et al. 2001). Briefly, a grid of equivalent sampling fields was outlined on the muscle slice profile. To ensure that every part of the slice had an equal chance of being sampled, a bidimensional stereological sampling procedure was applied analysing equivalent fields placed at a

fixed distance from each other on the tissue slice, using the "Grid" function in Fiji (Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA).

The same approach was used to evaluate the neutrophil elastase staining by calculating with Fiji software the percentage of covered area (Area fraction %) per field for each section in the analysis.

3.6 WESTERN BLOTTING

3.6.1 TISSUE COLLECTION

Mice were anaesthetised with a mix of ketamine (1.75mg/Kg) and medetomidine (1mg/Kg) and transcardially perfused with 50ml of 0.1M PBS pH7.4. Following the blood removal, the skeletal muscles (TA, GCM, GM and TB) were dissected out and immediately frozen in cooled isopentane. The spinal cord was fluxed from the vertebral column employing sterile physiological solution (0.9% NaCl) and dissected in the three main segments (i.e. cervical, thoracic and lumbar). Spinal cord segments and nerve were immediately frozen on dry ice. All tissues collected were stored at -80°C until required.

3.6.2 PROTEIN EXTRACTION AND QUANTIFICATION

First, 40µm thickness sections of frozen muscles were obtained with a cryostat (Leica Biosystems, Wetzlar, Germany). Spinal cord segments or muscle cryosections were homogenised in boiling in 0.1% sodium dodecyl sulfate (SDS) in distilled water solution with Teflon potter. Then, samples were boiled at 100°C for 5 minutes to facilitate the action of SDS, sonicated three times per 10 seconds and boiled at 100°C for 5 minutes to completely homogenise the tissue. Finally, the suspension was centrifuged at 1200g for 10 minutes and the supernatant collected.

Sciatic nerves were ground in the recovery vial in the presence of liquid nitrogen to obtain a fine powder. Immediately after grinding, the nerve powder was homogenised with Teflon potter in ice-cold homogenisation buffer (20mM Tris-HCl pH 7.4, 2% Triton X-100, 150mM NaCl, 1mM EDTA, 5mM MgCl2, 10% anhydrous glycerol, protease and phosphates inhibitor cocktail, Roche). Then samples were sonicated three times per 10 seconds and centrifuged at 1200g for 10 minutes at 4°C. Finally, the supernatant was collected and stored at -80°C unit use.

Protein extracts were quantified using the Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin (BSA) as standard. The absorbance of the solution was read at 562nm wavelength using the Infinite®200 multimode reader (Tecan). A simple linear regression analysis of the BSA curve was performed to which the absorbance of samples was interpolated to estimate the protein concentration of samples.

3.6.3 MONO-DIMENSIONAL SDS-POLY-ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Prior to electrophoresis, samples were boiled in SDS sample buffer (6% SDS, 0.1M DTT, 20% glycerol, 0.125M Tris/HCl pH6.8, 0.025% blue bromophenol) at 95°C for 5 minutes. Equal amounts of total proteins (20-30µg) were separated on Tris-glycine polyacrylamide gels and electroblotted onto a polyvinylidene difluoride (PVDF) membrane using a BioRad mini-transfer system (BioRad Laboratories, Hercules, CA, USA).

To quantify the total protein electroblotted, membranes were briefly immersed in Ponceau S solution (Merck Life Science) and rinsed in water. Membranes were then placed between plastic sheets, scanned on Epson Perfection 1260 scanner, and the densitometry analysis was performed with Image Lab 6.1 software (BioRad).

For the immunoblotting protocol, membranes were incubated in blocking buffer composed of 5% BSA dissolved in 0.1% Tween20 in Tris-buffered saline pH 7.4 (TBS-T) solution for 1h and then probed over-night at 4°C with the primary antibody diluted in 3% BSA in TBS-T. After three washes of 5 min in TBS-T, membranes were incubated with the opportunely diluted HRP-conjugated secondary antibody (Santa Cruz) for 1h at room temperature (RT) and finally washed three times per 5 min in TBS-T. Immunoreactivity was visualised with Luminata Forte Western HRP substrate (Millipore, Billerica, MA, USA) at ChemiDoc XRS (Biorad).

The optical density of the blots was measured with Image Lab 6.1 software (BioRad) and normalised to the total amount of protein loaded stained with Ponceau S solution (Thacker et al. 2016) unless otherwise specified. Results were expressed as the percentage in respect of the non-transgenic littermates.

The antibodies used in this study are listed below.

Antibody	Host species	Dilution	Supplier
Arginase 1 (Arg1)	rabbit	1:1˙000	Abcam
Beta importin (β importin)	rabbit	1:5˙000	Merck Millipore
Glial Fibrillary Acidic Protein (GFAP)	mouse	1:30˙000	Merck Millipore
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	mouse	1:10˙000	Merck Millipore
Green fluorescent protein (GFP)	chicken	1:5˙000	Merck Millipore
Heme binding subunit of NADPH oxidase ($gp91^{PHOX}$)	mouse	1:1˙000	BD
inducible Nitric Oxide Synthase (iNOS)	rabbit	1:1˙000	Invitrogen
Ionised calcium-binding adapter molecule 1 (Iba1)	rabbit	1:1˙000	Fujifilm Wako
Macrosialin (CD68)	rat	1:300	Biorad
Mannose receptor (CD206)	rabbit	1:500	Abcam
Musclin	goat	1:300	R&D Biosystems
Myelin Basic Protein (MBP)	rat	1:1˙000	Biorad
Myogenic determination gene (MyoD)	rabbit	1:5˙000	DSHB
Myogenic factor 4 (MyoG)	mouse	1:130	DSHB
Neural Cell Adhesion Molecule (NCAM)	rabbit	1:1˙000	Merck Millipore
Neurofilament heavy polypeptide (NF200)	rabbit	1:4˙000	Abcam
p75 neurotrophin receptor ($p75^{NTR}$)	goat	1:1˙000	Santa Cruz
Paired box 7 (Pax7)	mouse	1:1˙000	DSHB
Sirtuin 1 (SIRT1)	mouse	1:750	Sigma Aldrich

Table 11: *List of the antibodies used for the western blotting analysis.*

3.7 REAL-TIME PCR (RT-PCR)

3.7.1 TISSUE COLLECTION

Murine tissues were collected ad described in paragraph 4.6.1.

Human and muscle cryosections were collected in TRIzol™ (Invitrogen) and stored at -80°C until use.

3.7.2 RNA EXTRACTION AND cDNA SYNTHESIS

The total RNA from spinal cord, nerves and muscles was extracted using the TRIzol™ method (Invitrogen), purified with Ambion PureLink RNA columns (Thermo Fisher Scientific) according to the manufacturer's recommendation, and suspended in RNAse-free water. Extracted RNA was

quantified with Nanodrop™ Spectrophotometers (Thermo Fisher Scientific). Prior to the retro-transcription, RNA samples were treated with DNase I (Invitrogen) to avoid genomic DNA contamination, and the reverse transcription was done with the High Capacity cDNA Reverse Transcription Kit (Invitrogen).

The quality of the cDNA obtained was tested with a qualitative PCR using primers for the murine Superoxide Dismutase 2 (SOD2) gene (forward: TGCACTGAAGTTCAATGGTGG; reverse: TAGAGCAGGCAGCAATCTGT) or the human Peptidylprolyl Isomerase A (PPIA) gene (forward: GTCTCCTTCGAGCTGTTTGC; reverse: AGCCAAATCCTTTCTCTCCAG).

3.7.3 GENE EXPRESSION ANALYSIS

For Real-time PCR, the TaqMan™ Gene expression assay (Thermo Fisher Scientific) was employed following the manufacturer's instructions, on cDNA specimens in triplicate, using SensiFAST Probe Hi-ROX Kit (Aurogene) and 1X mix containing the specific probes (Thermo Fisher Scientific).

The TaqMan™ probes used in this study are listed below.

Probe	ID
Acetylcholine Receptor gamma subunit (AChRγ)	Mm00437419_m1
Beta-actin (β actin)	Mm02619580_g1
CD4	Mm00442754_m1
CD8a	Mm01182107_g1
Forkhead box P3 (FOXP3)	Mm00475162_m1
Insulin-like Growth Factor 1 (IGF1)	Mm00439560_m1
Interleukin 1 beta (IL1β)	Mm00434228_m1
Interleukin 4 (IL4)	Mm00445259_m1
Macrosialin (CD68)	Mm03047343_m1
Monocyte Chemoattractant Protein 1 (MCP1)	Mm00441242_m1
Tumour Necrosis Factor alpha (TNFα)	Mm00443258_m1
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	Hs02786624_g1
Monocyte Chemoattractant Protein 1 (MCP1)	Hs00234140_m1

Table 12*: List of the TaqMan™ probes used for the Real-Time PCR assay.*

Relative quantification was calculated from the ratio between the cycle number at which the signal crossed a threshold set (Ct) within the logarithmic phase of the given gene and that of the reference β-actin/GAPDH gene.

Mean values of the triplicate results for each animal were used as individual data for the Livak statistical analysis ($2^{-\Delta\Delta Ct}$). Conversely, the mean values of the triplicate resulted from the assessment of the human bioptic samples were analysed by variation of the Livak Method (i.e. $2^{Ct(\text{reference gene})-Ct(\text{target gene})}$) due to the absence of the "calibrator" (i.e. healthy control subjects) as described in the "*Real-Time PCR Application Guide*" (Biorad).

3.8 STATISTICAL ANALYSIS

All the statistical analyses were performed using Prism 7 for Windows (GraphPad Software Inc.). Values are reported as mean ± SEM.

Parameters (body weight and locomotor ability) used to evaluate disease progression in $SOD1^{G93A}$ mice were analysed by repeated-measures ANOVA followed by Sidak's post hoc test. Symptoms onset was analysed by Log-rank Mantel-Cox test and Kaplan-Meier plots were generated.

Satellite cells dynamic was analysed by two-way ANOVA followed by Tukey's Multiple Comparison Test.

Previous D'Agostino & Pearson omnibus normality test, mean values ± standard deviation were used for statistical analysis by Student's t-test for two groups or by One-way ANOVA followed by Tukey's multiple comparison test for more than two groups.

The non-parametric Spearman rank correlation was used to the bivariate analysis of the human samples.

For all analyses, a p-value < 0.05 was considered statistically significant. The asterisk * indicates the comparison with the non transgenic littermates, while the dot ° indicates the comparison between scAAV9_MCP1 treated mice and the control group (i.e. empty vector).

RESULTS

Chapter IV

Evaluation of the contribution of the immune response in the skeletal muscles of fast and slow progressing ALS patients

4.1 BACKGROUND and AIM

The skeletal muscle represents the first body compartment in which the ALS-related dysfunction appears. Indeed, progressive weakness and atrophy of skeletal muscles is the cardinal feature of the disease.

Although the muscle involvement in nourishing the degenerative phenomenon of ALS is still elusive, there is compelling evidence suggesting that it might fulfil a critical role in the disease (Loeffler et al., 2016). Indeed, preclinical and *in vitro* studies demonstrated that the specific expression of the mSOD1 within the skeletal muscle led to limb weakness, NMJ abnormalities, axon degeneration and glial cells activation, suggesting a direct role of muscles in ALS pathophysiology (Dobrowolny et al., 2008; Maimon et al., 2018; Wong and Martin 2010). Moreover, molecular signalling that regulates muscle reinnervation, regeneration (i.e. myogenic programme) and metabolism have found dysregulated in ALS patients (Elf et al., 2014; Di Pietro et al., 2018; Jensen et al., 2016; Pansarasa et al., 2014) and models (Scaricamazza et al., 2020; Pansarasa et al., 2014; Dobrowolny et al., 2018; Palamiuc et al., 2015; Manzano et al., 2013).

The knowledge recently acquired highlighted the pivotal role of the immune cell-mediated inflammation (Tidball and Villalta 2010; Tidball 2017; Howard et al., 2020; Pizza 2008; Sass et al., 2018), mainly driven by macrophages (Chazaud 2020), in the mechanisms underlying the muscular healing upon an injury. Therefore, the role fulfilled by the peripheral immune response in ALS muscle pathology is only starting to emerge.

We recently reported the reduced macrophages infiltration in the hind limb skeletal muscle of fast progressing compared with slow progressing $SOD1^{G93A}$ mice (Vallarola et al., 2018), highlighting the importance of the peripheral immune response in driving the speed of the disease progression. Therefore, the first section of this Thesis aimed to verify the heftiness of our preliminary observations through the characterisation of the extent in the activation of the peripheral immune response and the eventual inflammatory milieu established in skeletal muscle biopsies derived from fast and slow progressing sporadic ALS patients. Indeed, a detailed understanding of the muscle

pathology in ALS might lead to the identification of novel prognostic/therapeutic targets useful in clinical practice.

4.2 EXPERIMENTAL DESIGN

Muscles biopsies of the right quadriceps femoris or left vastus lateralis muscle from n=10 fast progressing (ΔFS>0.68) and n=10 slow progressing (ΔFS<0.68) age and sex-matched sporadic ALS patients, kindly provided by Dr Sorarù and Dr Pegoraro (Università degli Studi di Padova), have been analysed through a biochemical (western blotting) and gene expression approach (qRT-PCR). A more detailed list of the human samples analysed with the relative specifications is available in section 3.4 of material and methods.

4.3 CHARACTERISATION OF THE IMMUNE CELLS INFILTRATE AND ITS INFLAMMATORY FINGERPRINT IN THE SKELETAL MUSCLES OF ALS PATIENTS

To assess a striking correlation between the ΔFS and the skeletal muscle preservation, we started our characterisation analysing the expression of musclin, a myokine produced by glycolytic muscle fibres (Banzet et al., 2007). Studies in musclin deficient mice described this protein as an exercise-responsive factor promoting mitochondrial biogenesis and exercise endurance (Subbotina et al., 2015). Moreover, the induction of musclin was effective in reducing the muscle wasting in C26-bearing mice, a model of cancer cachexia (Re Cecconi et al., 2019).

Our analysis showed an inverse relationship between the expression level of musclin and the speed of the disease progression (Fig. 13A), suggesting the preservation of the fibres from the metabolic switch (fast to slow) (Telerman-Toppet and Coërs 1978) and reduced atrophy of the skeletal muscle in slow progressing compared with fast progressing ALS patients.

We next evaluated the extent of MCP1 activation within the human biopsies. Our analysis did not show any significant correlation between the chemokine transcription and the ΔFS score (Fig. 13B). Accordingly, the monocytes recruitment was unchanged in the two cohorts of patients, as demonstrated by the analysis of the monocytic Iba1 marker (Fig. 13C). Nevertheless, in line with our preclinical observation (Vallarola et al., 2018), we recorded an increased infiltration of activated macrophages in the skeletal muscle of slow progressing ALS patients as demonstrated by the

inverse correlation between the expression level of the CD68 phagocytic marker and the ΔFS score (Fig. 13D).

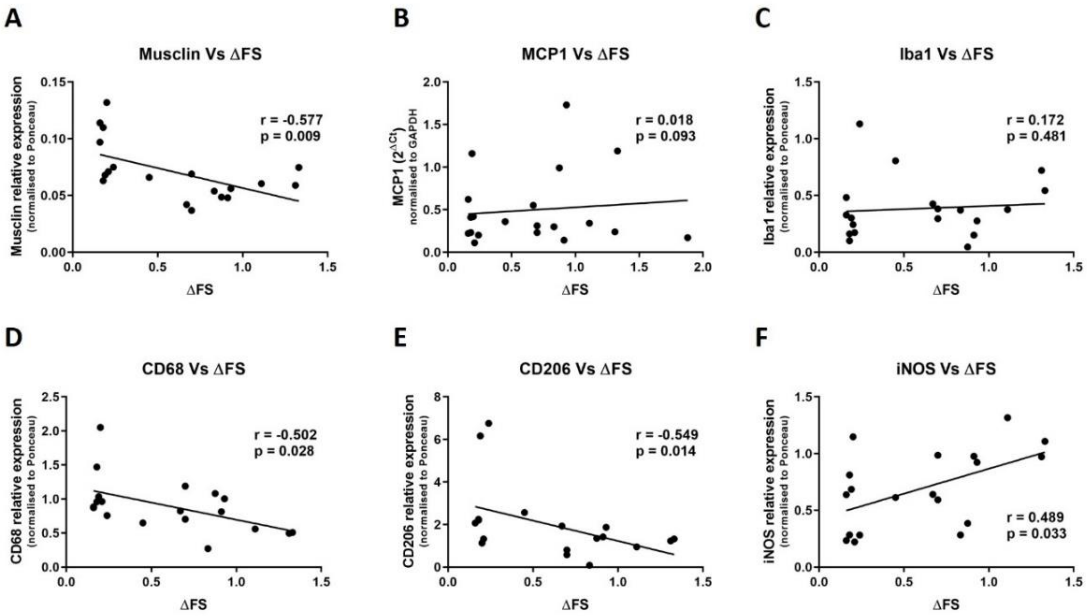

Figure 13: *Bivariate analysis to measure the strength of association between the muscular expression of Musclin (A), MCP1 (B), Iba1 (C), CD68 (D), CD206 (E) and iNOS (F) and the ΔFS score of ALS patients. Data were analysed by non-parametric Spearman rank correlation.*

Preclinical evidence showed that the phenotypic macrophage switching (M1->M2) is a fundamental step to trigger and achieve the tissue regeneration upon an injury (Arnold et al., 2007; Patsalos et al., 2017). Therefore, we characterised the macrophages inflammatory fingerprint in the skeletal muscles of fast and slow progressing ALS patients analysing the expression of the mannose receptor (CD206) and the inducible nitric oxide synthase (iNOS), as markers of the M2 and M1 macrophage polarisation, respectively (Novak and Koh 2013). The data obtained revealed an inverse relationship between the expression of the M2 marker CD206 and the speed of the disease progression (Fig. 13E). Specularly, the expression of the M1 marker iNOS positively correlated with the faster disease progression of human ALS (Fig. 13F).

4.4 DISCUSSION

ALS is a neuromuscular disease. Indeed, the most common symptoms that appear in both familial and sporadic patients are muscle weakness, twitching, and cramping, which eventually can lead to

the impairment of muscles function (Wijesekera and Leigh 2009). However, the contribution of skeletal muscle in nourishing the degenerative phenomenon of ALS is still elusive.

Recent evidence highlighted the pivotal role of leucocytes, particularly macrophages, in governing and promoting the regenerative response of the skeletal muscle upon acute injury (Tidball 2017; Howard et al., 2020; Chazaud 2020). However, the contribution of the peripheral immune response has been so far underestimated in ALS, although immune cells infiltration has been reported within the skeletal muscle of both ALS patients and disease models (Jensen et al., 2016; Chiu et al., 2009). For the first time, here we reported an inverse correlation between the speed of the disease progression and the extent of $CD68^{+}$ activated macrophages within the skeletal muscles of ALS patients, suggesting the protective role of these immune cells even during the chronic damage occurring in ALS. Nevertheless, we did not find any difference between the two cohorts of patients in the expression of MCP1 chemokine, indicating that the extent of haematogenous monocyte recruitment was unchanged between fast and slow progressing ALS patients.

Noteworthy, our analysis sustains the preclinical evidence describing the phenotypic transition (M1->M2) of macrophages as a fundamental step to promote the muscle healing upon an acute injury (Arnold et al., 2007; Patsalos et al., 2017). Indeed, the CD206 anti-inflammatory marker was highly expressed in the skeletal muscle of slow progressing ALS patients. In contrast, the infiltrated macrophages of fast progressing patients exhibited a proinflammatory fingerprint as demonstrated by the higher expression of iNOS. Accordingly, the activation of the musclin myokine was higher in the slow progressing ALS patients, indicating a preservation of the skeletal muscle compared with fast progressing patients.

Although preliminary, this evidence shed light on the importance of the peripheral immune response in counteracting the progressive degeneration occurring within ALS skeletal muscles. Moreover, given the accessibility in the collection of the muscular bioptic samples, even longitudinally in the disease course, the analysis of the immune muscle profile might be useful as a clinical adjunct in the prognostic evaluation of ALS patients.

RESULTS

Chapter V

Selection of the best route of administration of the scAAV9_GFP vector to target the motor unit in ALS mice

5.1 BACKGROUND and AIM

Therapeutic gene delivery to the CNS is a significant challenge for the treatment of neurodegenerative diseases (Joshi et al., 2017). Accordingly, the first step of this project was to select the best route of administration of the viral vector to obtain an efficient and accurate induction of MCP1 chemokine in ALS mice. Indeed, our preliminary evidence demonstrated that the MN of ALS mice expresses immune molecules (among which MCP1) and that their upregulation is more prominent in the animal model characterised by the slower disease progression (i.e. C57 $SOD1^{G93A}$) (Nardo et al. 2013). Moreover, according to the previous evidence demonstrating the importance of the MCP1/CCR2 axis in promoting the macrophages recruitment and thus the nerve regeneration upon injury (Siebert et al., 2000; Zigmond and Echevarria 2019), we recorded a significant expression of the chemokine alongside the motor axons of the slow progressing compared with fast progressing $SOD1^{G93A}$ mice (Nardo et al., 2016b). This evidence depicts the pivotal role of the central and peripheral immune response, particularly that mediated by the MCP1 chemokine, in regulating the speed of the disease progression of ALS mice.

Among the viral vectors available, we selected the self-complementary Adeno-Associated Virus (scAAV) which we believe is superior to other recombinant AAV for several properties including i) no need to convert a single-stranded genome in a double-stranded prior to expression, ii) stability, iii) efficient nuclear transport and iv) high gene expression (McCarty 2008). Notably, scAAV is the transfer vehicle with the most potential use in therapies of neuromuscular disorders with non-existent treatment options because of its safety profile and efficiency at transducing a wide range of cell types (Chtarto et al., 2013; Deverman et al., 2018). Among serotypes, we selected the scAAV9 because of its higher transduction efficiency in neurons through different routes of delivery (Li and Snider 2018; Dayton et al., 2012).

Based on recent evidence concerning the use of the AAV-mediated gene therapy in neurodegenerative diseases (Benkhelifa-Ziyyat et al., 2013; Duque et al., 2009; Foust et al., 2013; Perez et al., 2020), we tested the most commonly used routes of administration: a CNS-direct

delivery, i.e. intracerebroventricular (i.c.v.) injection, and systemic delivery, i.e. intramuscular (i.m.) injection.

5.2 EXPERIMENTAL DESIGN

To test the i.c.v. and i.m. routes of administration, the two strains of SOD1^{G93A} mice were treated with the vector opportunely engineered with the enhanced Green Fluorescent Protein reporter gene sequence (scAAV9_GFP) (purchased by Virovek Inc., as described in section 3.3.1) under CMV (cytomegalovirus) promoter to analyse the transduction efficiency and the distribution within the mouse body compartments.

For the evaluation of the transduction efficiency following the CNS-direct administration, ALS mice (n=6 per group) underwent i.c.v. injection of 4.48x10^{12}vg/µL scAAV9_GFP at postnatal day 1 (P1) (Glascock et al., 2011; Gholizadeh et al., 2013) and the histological and molecular analyses of tissues were performed six weeks after the treatment.

Conversely, to increase its translatability to the clinical practice, the i.m. injection was tested in adult SOD1^{G93A} mice (n=6 per group) before the symptom onset. Mice underwent a single bilateral i.m. injection of a lower dose of scAAV9_GFP (2.18x10^{10}vg/µL) to reduce the immunogenicity deriving from the systemic administration (Gruntman et al., 2013). The scAAV9_GFP was injected in both hindlimb (*Gastrocnemius Caput Medialis*, GCM; *Tibialis Anterior*, TA and *Gluteus Maximus*, GM) and forelimb (*Triceps Brachii*, TB) muscles to target most segments of the spinal cord and relative MNs (Tosolini et al., 2013; Mohan et al., 2014). Mice were treated at 10 weeks of age and the analyses were performed three weeks later.

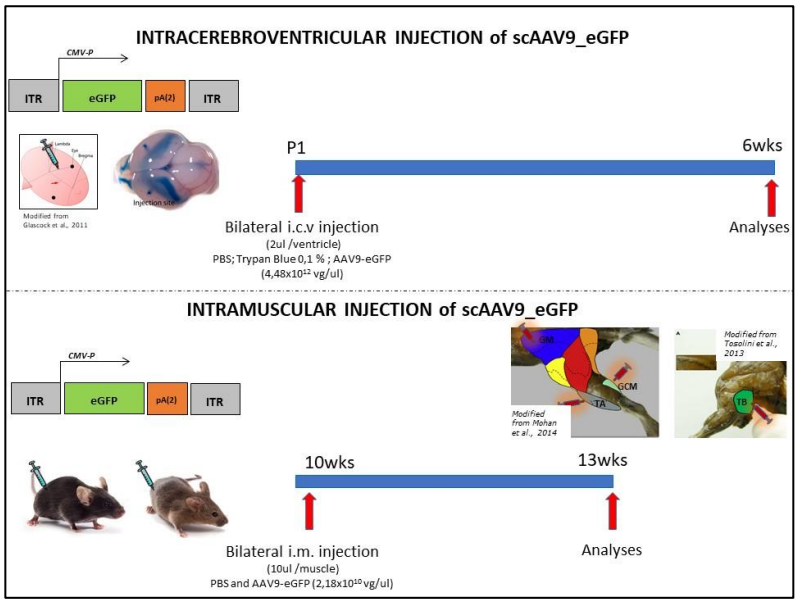

Figure 14: *Experimental schedule of i.c.v. and i.m. injection of scAAV9_GFP in fast and slow progressing SOD1^{G93A} mice.*

5.3 ANALYSIS OF GFP EXPRESSION ALONGSIDE THE MOTOR UNIT OF FAST AND SLOW PROGRESSING ALS MICE

5.3.1 ANALYSIS OF GFP EXPRESSION IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEM

To verify whether both routes of administration of scAAV9_GFP were able to transduce MN efficiently, the expression levels and the cellular distribution of the reporter gene were analysed in the CNS of ALS mice. According to previous evidence (Dirren et al. 2014; Benkhelifa-Ziyyat et al. 2013), both routes of administration tested were able to reach the CNS as demonstrated by the strong GFP expression recorded in the spinal cord of treated mice compared with the control group (untreated mice) (Fig. 15A-D, Fig. 16A-D'). However, the histological analysis performed showed that the i.m. injection of scAAV9_GFP specifically transduced the MN soma, as confirmed by the absent colocalisation between the reporter gene and astroglial markers (Fig. 16C-D'). Conversely, the CNS-direct delivery led to the transduction of both MNs and glial cells (Fig. 15D).

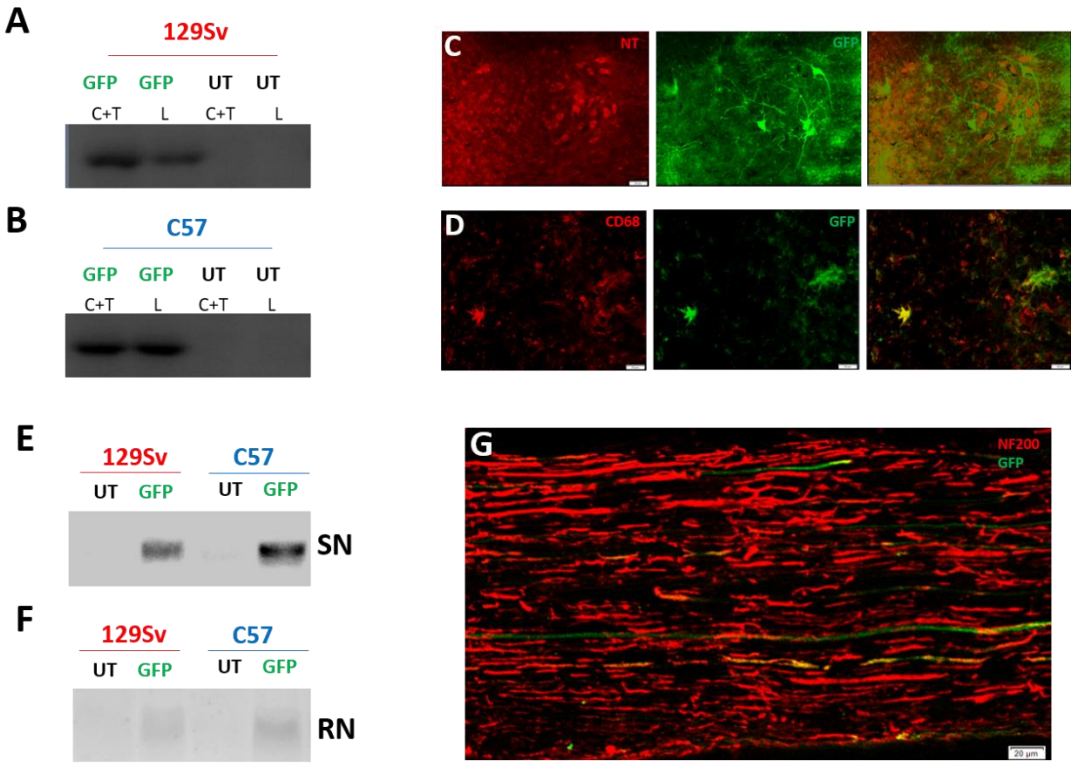

Figure 15: *Analysis of Green Fluorescent Protein (GFP) expression in the CNS and PNS of fast and slow progressing ALS mice following i.c.v. injection of scAAV9_GFP. (A, B, E, F) Representative immunoblot analysis of GFP in the spinal cord (A, B), sciatic (E) and radial (F) nerve of treated (GFP) and untreated (UT) mice (C+T, cervical and thoracic spinal cord; L, lumbar spinal cord; SN, sciatic nerve; RN, radial nerve; GFP). (C, D, G) Immunohistochemistry analysis of GFP expression in the spinal cord and sciatic nerve of ALS mice. Following i.c.v. treatment, GFP colocalised with motor neurons (NT, neurotrace) (C), microglia (CD68) (D) and motor axon (NF200, neurofilament) (G) Scale bar=20µm.*

ALS is a multisystemic disease and it has been recently defined as distal axonopathy (Moloney et al., 2014). Accordingly, recent evidence illustrated the importance of the PNS immune response in the disease progression of ALS patients (Schreiber et al., 2019). Moreover, we have recently demonstrated that the ability at activating the MCP1 pathway and recruiting immune cells within damaged nerves significantly influenced the speed of the disease progression in the SOD1^{G93A} model (Nardo et al., 2016b).

Therefore, we analysed the ability of the two delivery systems to target the motor axons. The western blot and immunohistological analysis showed a strong GFP expression within the sciatic and radial nerves of treated mice compared with the control group following both i.c.v (Fig. 15E-G) and i.m. (Fig. 16E-G) administration of scAAV9_GFP, confirming the ability of the viral vector to move along motor axons anterogradely (i.c.v injection) or retrogradely (i.m. injection), respectively (Castle et al., 2016).

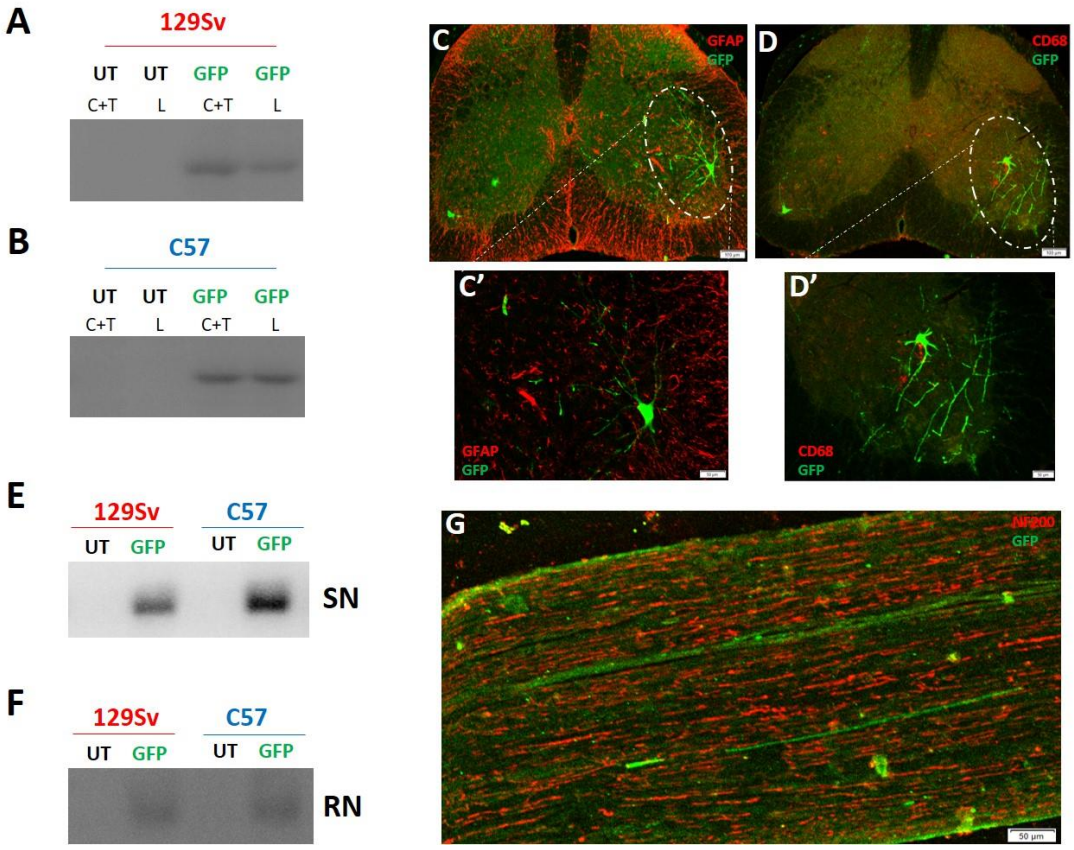

Figure 16: *Analysis of the Green Fluorescent Protein (GFP) expression in the CNS and PNS of fast and slow progressing ALS mice following i.m injection of scAAV9_GFP. (A, B, E, F) Representative immunoblot analysis of GFP in the spinal cord (A, B), sciatic (E) and radial (F) nerve of treated (GFP) and untreated (UT) mice (C+T, cervical and thoracic spinal cord; L, lumbar spinal cord; SN, sciatic nerve; RN, radial nerve). (C-D', G) Immunohistochemistry analysis of GFP expression in the spinal cord and sciatic nerve of ALS mice. Following i.m treatment, GFP is specifically expressed by motor neurons and motor axons (NF200, neurofilament) (G), but not astrocytes (GFAP) (C, C') and microglia (CD68) (D, D'). C', D' and G Scale bar= 50µm; C and D scale bar= 100µm.*

5.3.2 ANALYSIS OF GFP EXPRESSION IN THE PERIPHERAL ORGANS OF ALS MICE

Muscle weakness is considered the cardinal sign of ALS that appears before MN death. A debate still exists as to whether denervation originates from the neuron or the muscle (Dadon-Nachum et al., 2011; Dobrowolny et al., 2008) and regarding the involvement of muscle remodelling as an actor in ALS progression (Jensen et al., 2016). Moreover, MCP1 chemokine seems to be pivotal in regulating the regeneration of skeletal muscle (Shireman et al., 2007). Thus, verifying the capability of the two scAAV9 administration routes herein tested to target the skeletal muscles represents also a fundamental goal for the overall aim of this project.

As previously reported (Riaz et al., 2015), we confirmed the ability of the i.m. injection of scAAV9_GFP to transduce the muscle fibres significantly (Fig. 17C-F). Conversely, following i.c.v

injection we did not record any GFP signal in the TA and TB muscles, indicating the inability of the viral vector at transducing muscle fibres once injected in the CNS (Fig. 17A, B, E, F).

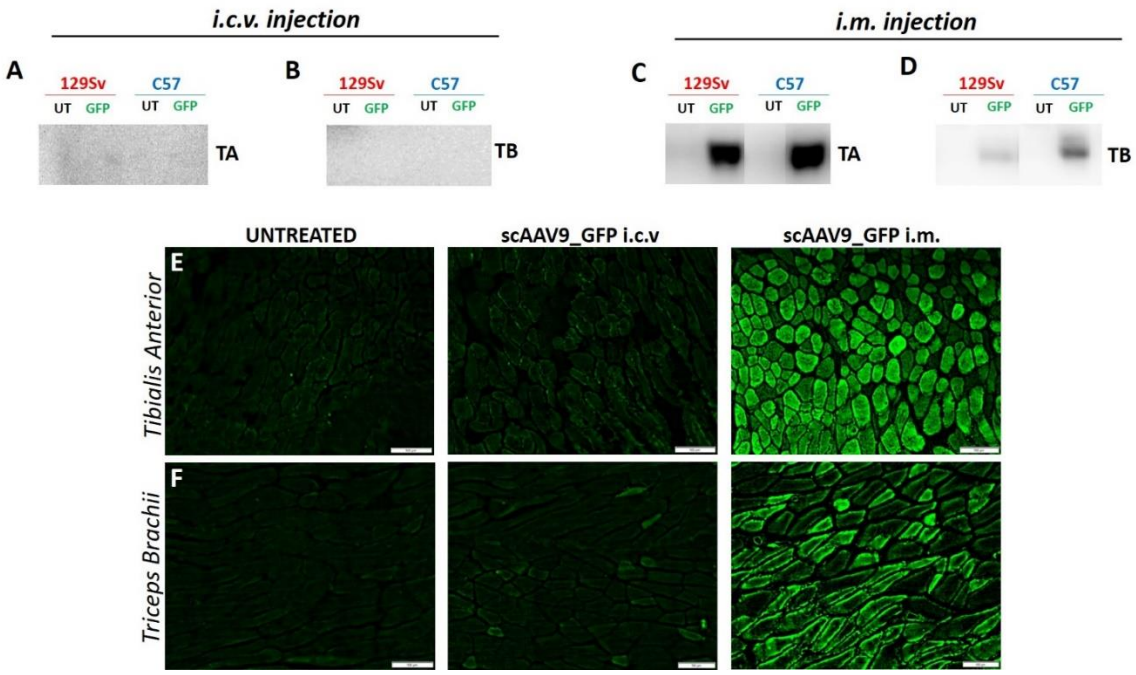

Figure 17: *Analysis of the Green Fluorescent Protein (GFP) expression in the Tibialis anterior (TA) and Triceps Brachii (TB) muscle of fast and slow progressing ALS mice following the i.c.v and i.m injection of scAAV9_GFP (GFP, scAAV9_GFP treated; UT, untreated). Representative immunoblot and confocal micrographs of the GFP expression in the TA and TB muscle following i.c.v (A, B, E, F) or i.m. (C, D, E, F) injection of the scAAV9_GFP. Scale bar= 100µm.*

Recent evidence reported that scAAV9 exhibits a specific tropism toward neurons, muscle, liver and lung (Castle et al., 2016). To avoid the manifestation of secondary side effects following the induction of a proinflammatory chemokine in whole mouse body, we analysed the GFP expression in the liver and lung. As shown in Figure 18, we did not record any GFP signal within lung either following i.c.v or i.m. injection of the scAAV9_GFP (Fig. 18B, C). Conversely, according to previous evidence (Dirren et al., 2014), the scAAV9 exhibited a great tropism toward the liver (Fig. 18A, D). Notably, our analysis revealed higher GFP expression in the liver following i.c.v compared with i.m. administration (Fig. 18E).

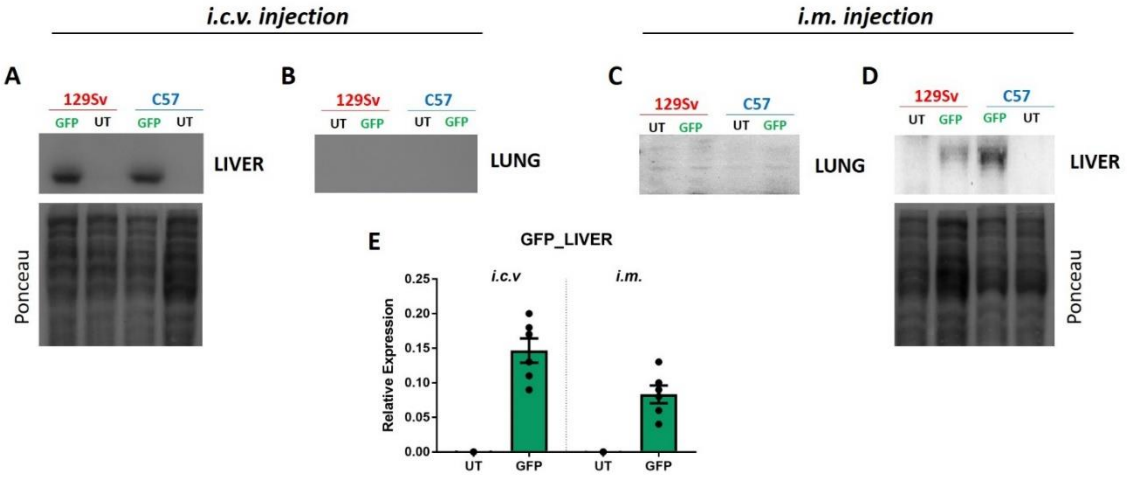

Figure 18: *Representative immunoblot analysis of the Green Fluorescent Protein (GFP) expression in the liver and lung of fast and slow progressing ALS mice following the i.c.v (A, B) and i.m (C, D) injection of scAAV9_GFP (GFP, scAAV9_GFP treated; UT, untreated). (E) The densitometric analysis revealed a higher expression of the reporter gene in the liver of i.c.v treated compared with i.m. treated mice (n=6 per group).*

5.4 DISCUSSION

The targeting of CNS is the major challenge for the application of gene therapy to neurodegenerative diseases (Joshi et al., 2017; Walthers and Seidlits 2015).

Here, two innovative gene delivery protocols have been tested with the aim to target the entire motor unit in $SOD1^{G93A}$ mice.

The data collected from the analysis of the GFP expression showed that, although the i.c.v. injection of scAAV9_GFP efficiently transduced the nervous system, the use of a promoter non-specific for the MNs (i.e. CMV promoter) led to the targeting also of glial cells, which are one of the primary sources of neurotoxic pro-inflammatory factors (including MCP1) in the CNS (Sargsyan et al., 2009). Conversely, the i.m. administration of scAAV9_GFP led to the specific transduction of MN perikarya and axons, avoiding the targeting of astroglia.

Notably, the CNS-direct administration of scAAV9_GFP was not able to transduce skeletal muscles. This deficit represents a black mark of this administration route compared with the i.m. injection since increasing evidence candidate the skeletal muscles as an emerging target for therapeutic interventions in ALS (Di Pietro et al., 2017; Loeffler et al., 2016; Shefner 2009; Musarò et al., 2019). Moreover, it has been reported the involvement of MCP1 and other inflammatory factors in triggering the muscle regeneration (Shireman et al., 2007; Martinez et al., 2010; Zhang et al., 2014a), candidating the targeting of skeletal muscles as a fundamental goal of this project.

Furthermore, the i.m. injection showed the lower intrinsic tropism of scAAV9 towards liver compared to the i.c.v administration (Castle et al., 2016), warding off the establishment of secondary side effects due to the undesired targeting.

Finally, the i.m. injection of scAAV has proven effective in the adult mouse, thus increasing the translational potential of this experimental protocol to the clinical practice.

In conclusion, the data herein collected designated the i.m. delivery as the optimal protocol to our purpose. Accordingly, the i.m. route of administration will be used for the induction of MCP1 chemokine in both fast and slow progressing ALS mice. The effects derived from the treatment will be described in the next sections of this Thesis.

RESULTS

Chapter VI

Evaluation of the effect of the i.m. injection of scAAV9_MCP1 on the disease progression and muscle impairment of fast and slow progressing ALS mice

6.1 BACKGROUND and AIM

MCP1 is considered one of the most potent inflammatory chemokine able to drive and exacerbate the phlogosis in several tissues (Gu et al., 1999). Although inflammation has so far considered a prejudicial process in the alteration of tissue homeostasis, it seems to be pivotal in wound healing and regeneration (Eming et al., 2017).

In keeping with this, our previous data showed a higher MCP1 expression in slow progressing compared with fast progressing SOD1^{G93A} ALS mice (Nardo et al. 2013; 2016b). Moreover, recent evidence reported a beneficial involvement of this inflammatory molecule within the main body compartments affected by ALS: MN (Locatelli et al., 2012; Conductier et al., 2010; Kwon et al., 2015), axon (Deng et al., 2015; Liu et al., 2019b) and skeletal muscle (Shireman et al., 2007; Martinez et al., 2010; Lu et al., 2011a).

Therefore, this project aimed to analyse whether the specific induction of MCP1 early in the disease is able to modify the disease progression and the pathological and molecular features in ALS mice. Particularly, in light of the higher MCP1 expression recorded in the MN soma and axons of slow progressing (C57 SOD1^{G93A}) compared with fast progressing (129Sv SOD1^{G93A}) ALS mice (Nardo et al. 2013; 2016b), this project aimed to investigate whether the sustained expression of the chemokine is sufficient to ameliorate the clinical outcome in C57 SOD1^{G93A} mice and if the chemokine induction in the animal model characterised by the faint activation of this immune axis (i.e. 129Sv SOD1^{G93A}) is effective to slow down the disease progression tangibly.

6.2 EXPERIMENTAL DESIGN

To induce the chemokine, a scAAV9 vector properly engineered with the murine sequence encoding for the *MCP1* gene (scAAV9_MCP1) under the CMV promoter was purchased by Virovek Inc. (as described in section 3.3.1).

The same protocol used for the i.m. injection of scAAV9_GFP (paragraph 5.2) was applied to induce MCP1 in the two ALS models. Fast (n=12) and slow (n=10) progressing ALS mice underwent a single bilateral i.m. injection of 2.18x10^{10}vg/µL scAAV9_MCP1 in both hindlimb (TA, GCM and GM) and

forelimb (TB) muscles (10µL per muscle) at 8 weeks of age (pre-symptomatic stage of the disease). An empty vector was used as control.

To evaluate the effect of MCP1 induction on the disease onset and progression, behavioural tests were performed starting from 8 weeks until the symptomatic stage of the disease (i.e. 17weeks, fast progressing 129Sv SOD1^{G93A} mice; 20weeks, slow progressing C57 SOD1^{G93A} mice).

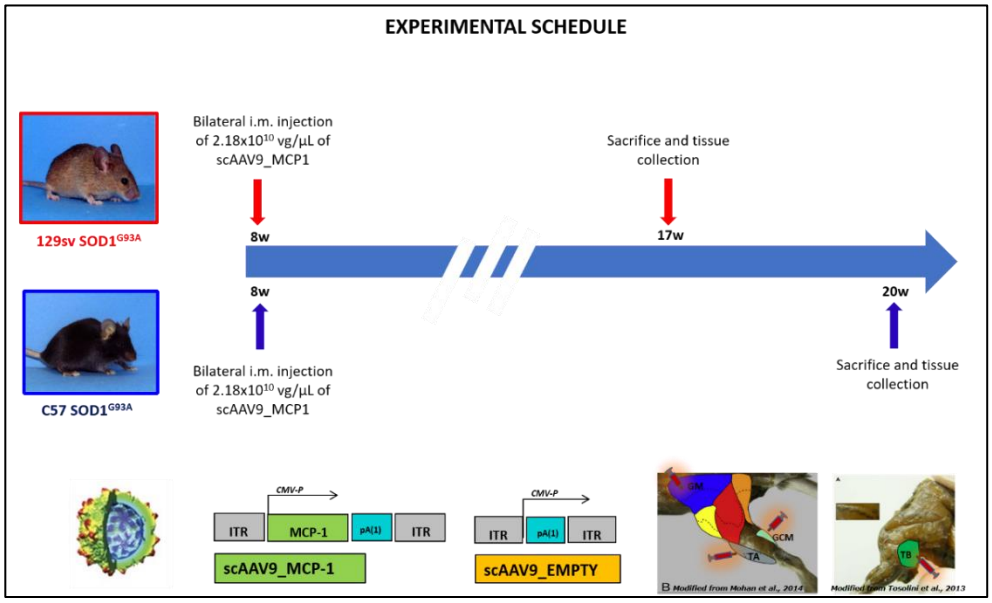

Figure 19: *Experimental schedule of i.m. injection of scAAV9_MCP1 in fast and slow progressing SOD1^{G93A} mice.*

6.3 ANALYSIS OF THE EFFECT OF MCP1 INDUCTION ON THE MOTOR IMPAIRMENT AND DISEASE PROGRESSION OF FAST AND SLOW PROGRESSING ALS MICE

Aimed to assess whether MCP1 induction could have any effect on the determination of the disease onset and progression, the muscle force impairment and the weight loss were monitored twice a week in ALS mice starting from 8 weeks of age until their respective symptomatic stage of the disease.

In both ALS models, the MCP1 induction did not exert any significant effect in terms of body weight loss compared to the control group (Fig. 20A, B), suggesting that neither the i.m. injection of scAAV9 nor the induction of a pro-inflammatory chemokine within motor unit causes any macroscopic adverse effect in SOD1^{G93A} mice.

Surprisingly, the behavioural analysis showed a different effect of the treatment on the motor ability of the two ALS strains. Although the treatment did not modify the disease onset of fast

progressing mice (Empty 14.3±1.6 weeks, MCP1 13.5±1.4 weeks; p=0.3455) (Fig. 20C), we recorded a worsening of the motor performance in the scAAV9_MCP1 treated mice compared with the control group in the later stage of the disease (Fig. 20B).

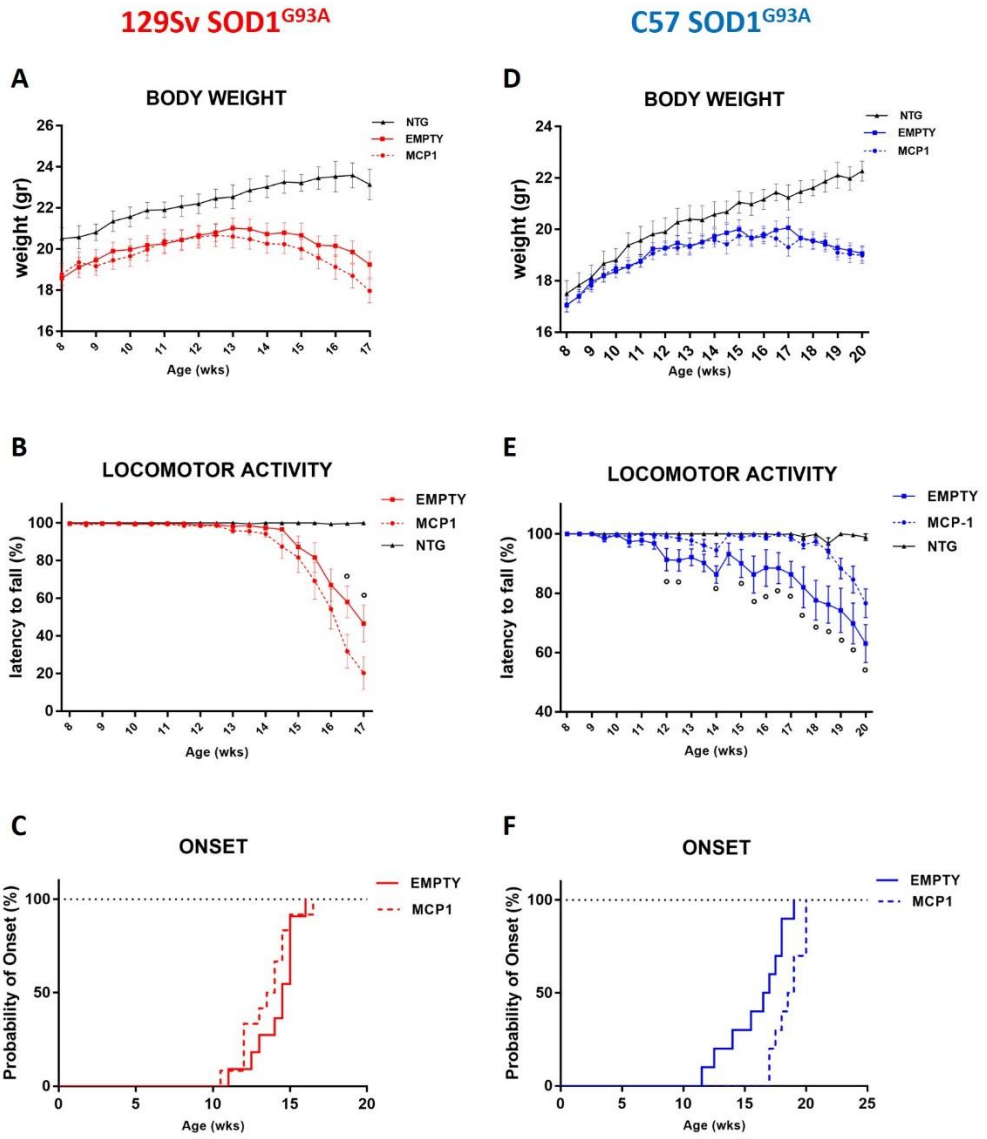

Figure 20: *Behavioural analysis of fast progressing (A-C) and slow progressing mice (D-F) treated i.m. with scAAV9_MCP1 compared with the control group (empty vector) (n=12/group, 129Sv SOD1^{G93A} mice; n=10/group, C57 SOD1^{G93A} mice). The i.m. injection of scAAV9_MCP1 did not affect the body weight in both strains of ALS mice (A, D). The scAAV9_MCP1 injection increased the muscle force impairment in the fast progressing mice (B), while improved the motor performance of the slow progressing mice (E). Data are reported as mean±SEM for each time point. °p< 0.05 EMPTY Vs MCP1 by repeated-measures ANOVA with Sidak's post-analysis. The scAAV9_MCP1 injection did not modify the onset of the disease in fast progressing mice (C), while postponed the appearance of the muscle force deficit in slow progressing mice (F). Data are expressed as mean±SEM by Mantel-Cox Log Rank Test.*

Conversely, in slow progressing mice, the chemokine induction significantly slowed down the disease course. Indeed, in the scAAV9_MCP1 treated mice, we recorded an amelioration of the motor ability since the early phase of the disease (Fig. 20E), and this resulted in the postponement of the motor symptom onset of ~2weeks compared with the control group (Empty 16±2.5 weeks,

MCP1 18.6±1.2 weeks; p=0.0088) (Fig. 20F). Notably, the induction of a pro-inflammatory chemokine did not basally affect animals' motor performance, as demonstrated by the ability of both strains of non-transgenic scAAV9_MCP1 treated mice to pass the behavioural test throughout the analysis (Fig. 20B, E).

6.4 VALIDATION OF MCP1 INDUCTION IN THE SKELETAL MUSCLES OF FAST AND SLOW PROGRESSING ALS MICE

To assess whether the different clinical outcome observed in the two ALS mice following the treatment was directly associated to the induction of MCP1 and not the result of an intrinsic variability of each mouse strain to the scAAV9 vector (He et al., 2019), the transcription level of the chemokine was evaluated in TA and TB muscles. As expected, MCP1 transcript was significantly upregulated within the skeletal muscles of both strains of $SOD1^{G93A}$ treated mice compared with their respective control groups, albeit to a lower extent in the forelimb compared with hindlimb muscles (Fig. 21).

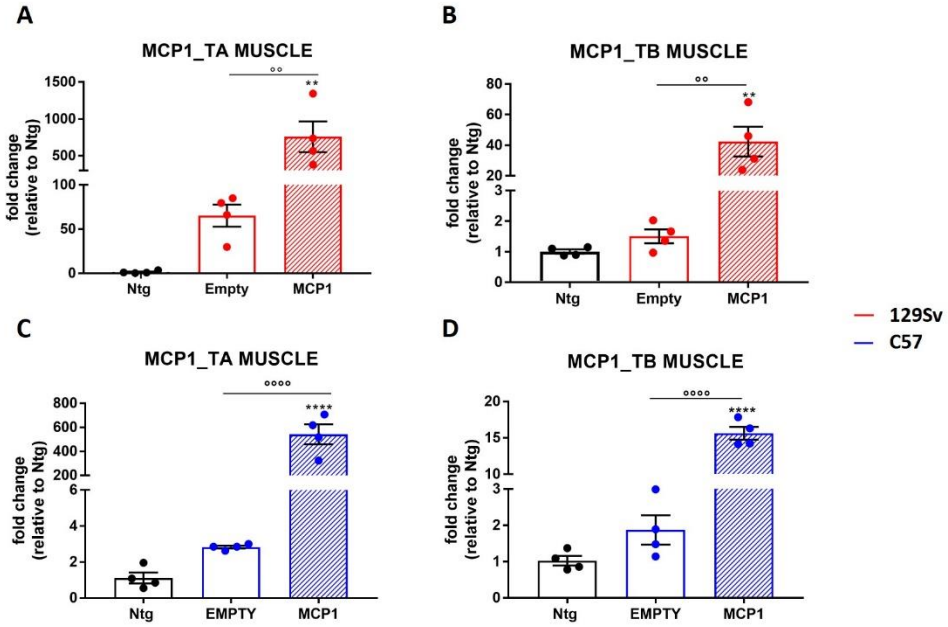

Figure 21: *Real-Time PCR analysis of MCP1 transcript in the tibialis anterior (TA) and triceps brachii (TB) muscle of 129Sv $SOD1^{G93A}$ (A, B) and C57 $SOD1^{G93A}$ mice (C, D) compared with their respective non-transgenic (Ntg) littermates. Following scAAV9_MCP1 injection, the chemokine transcript resulted dramatically upregulated in treated mice compared with the control groups (Fold change vs Ntg TA muscle_129Sv $SOD1^{G93A}$: 65.08±12.4 Empty, 757.1±208.5 MCP1; C57 $SOD1^{G93A}$: 2.97±0.2 Empty, 542.1±82.5 MCP1. Fold change vs Ntg TB muscle_129Sv $SOD1^{G93A}$: 1.50±0.2 Empty, 42.31±9.7 MCP1; C57 $SOD1^{G93A}$: 1.87±0.4 Empty, 16.13±1.0 MCP1). Data are normalised to β-actin and expressed as mean±SEM (n=4 per experimental group). **p<0.01, ****p<0.0001 Ntg vs MCP1; °°p<0.01, °°°°P<0.0001 EMPTY vs MCP1 by ANOVA with Tukey's post-analysis.*

6.5 ANALYSIS OF DENERVATION ATROPHY OF THE SKELETAL MUSCLES OF FAST AND SLOW PROGRESSING ALS MICE

Aimed to characterise the different effect recorded in the motor performance of the two SOD1^{G93A} models following MCP1 induction, we started our investigation from the skeletal muscles. In fact, besides being the injection site of scAAV9_MCP1, the impairment of skeletal muscles is an early event in the disease pathology (Loeffler et al., 2016; Campanari et al., 2016) pivotal in determining the motor ability and thus the overall survival of ALS mice. Therefore, the characterisation of the muscle atrophy and denervation could provide useful information about the different effect observed in fast and slow progressing SOD1^{G93A} mice following MCP1 induction.

Among the injected muscles of the hind limbs, we focused our attention on the TA muscle considering the higher percentage of fast-fatigable muscle fibres than the GCM (Lionikas et al., 2005). Indeed, skeletal muscles of ALS patients and models show a progressive and irreversible metabolic switch from fast-glycolytic to slow-oxidative muscle fibres composition (Dobrowolny et al., 2018; Telerman-Toppet and Coërs 1978; Palamiuc et al., 2015). Therefore, the analysis of the TA muscle would have been more informative regarding the changes that occurred during the disease progression. In parallel, the attention was focused on the injected forelimbs TB muscle.

The analysis of muscles weight did not show any difference in the TA muscle wasting between treated mice and control group in both strains of ALS mice (129Sv SOD1^{G93A}: 27.5±4.1% Empty, 35.6±5.1% MCP1; C57 SOD1^{G93A}: 50.7±4.5% Empty, 52.7±4.7% MCP1) (Fig. 22A, B). Conversely, the forepaw muscle appeared protected in the C57 SOD1^{G93A} (45.8±4.6% Empty, 33.4±3.2% MCP1) but not 129Sv SOD1^{G93A} mice following the chemokine induction (22.2±4.8% Empty, 30.9±4.9% MCP1) (Fig. 22C, D).

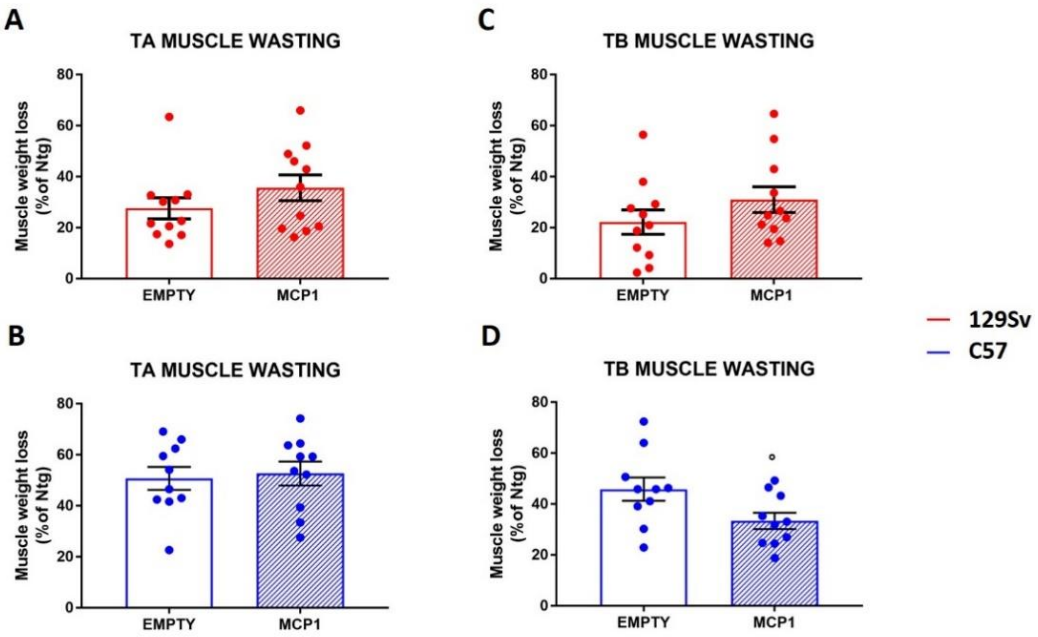

Figure 22: *Analysis of the wasting of the tibialis anterior (TA) and triceps brachii (TB) muscles in 129Sv SOD1^{G93A} (A, C) and C57SOD1^{G93A} (B, D) mice. Data are expressed as mean±SEM (n=12 per group, 129sv SOD1^{G93A}; n=10 per group, C57 SOD1^{G93A}) by unpaired t-test.*

Previous experiments have shown an alteration in the synthesis of the acetylcholine receptor (AChR) subunits during the denervation of the neuromuscular junction (NMJ), characterised by the replacement of the adult epsilon subunit (AChRε) with the foetal gamma subunit (AChRγ) (Rimer et al., 1997; Dobrowolny et al., 2011). Therefore, we analysed the transcription level of AChRγ in the TA and TB muscles of symptomatic ALS mice upon MCP1 induction as an index of NMJ denervation. The analysis revealed that the transcription level of AChRγ was significantly reduced by the scAAV9_MCP1 injection in the TA muscle of fast progressing mice (Fig. 23A). In contrast, no difference was recorded between treated and control C57 SOD1^{G93A} mice within the hind limb muscle (Fig. 23B). Conversely, upon MCP1 induction, the forepaw muscle of 129Sv SOD1^{G93A} mice was significantly denervated, whereas marked protection of the TB muscle NMJs was recorded in C57 SOD1^{G93A} mice compared with their respective controls (Fig. 23C, D).

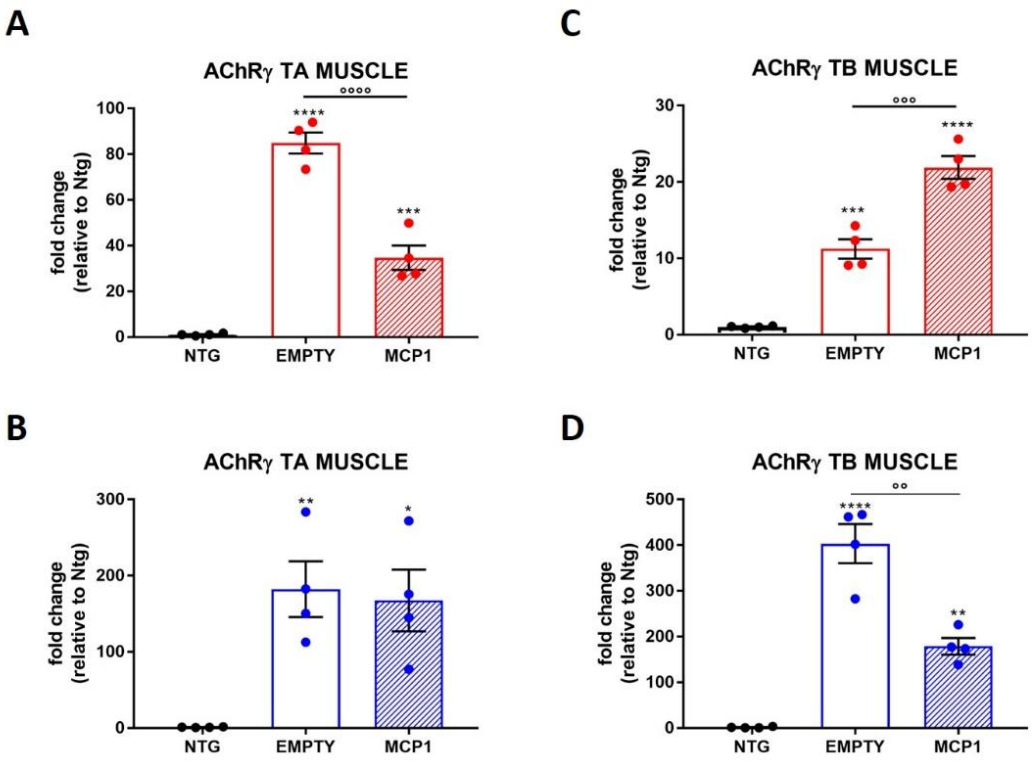

Figure 23: *Real-time PCR analysis of the gamma subunit of the acetylcholine receptor (AChRγ) in the tibialis anterior (TA) and triceps brachii (TB) muscles of 129Sv SOD1^{G93A} (A, C) and C57 SOD1^{G93A} mice (B, D). Data are normalised to βactin and expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Ntg Vs EMPTY or MCP1; °° p<0.01, °°° p<0.001, °°°° p<0.0001 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

To better characterise the NMJ degenerative phenomenon in the two ALS strains upon MCP1 induction, we next analysed the expression of the Neural Cell Adhesion Molecule (NCAM), a glycoprotein involved in neuron-muscle adhesion which is accumulated by denervated or paralysed skeletal muscle (Covault and Sanes 1985; Hegedus et al., 2007).

The immunoblot analysis did not show any significant difference in the NCAM expression in the TA and TB muscles between the treated and control group in both strains of ALS mice (Fig. 24). However, upon MCP1 induction, the two strains of ALS mice showed an opposite trend in the NCAM expression, suggesting a different effect of the treatment in the muscular compartment of the two SOD1^{G93A} models. Accordingly, the evaluation of the NMJ degenerative phenomenon assessing, the overlap between the pre-synaptic (stained with synaptic vesicle protein 2 and neurofilament) and post-synaptic (stained with fluorescent α-bungarotoxin) domain of the AChR (Sleigh et al. 2014) confirmed the immunoblot analysis (data not show).

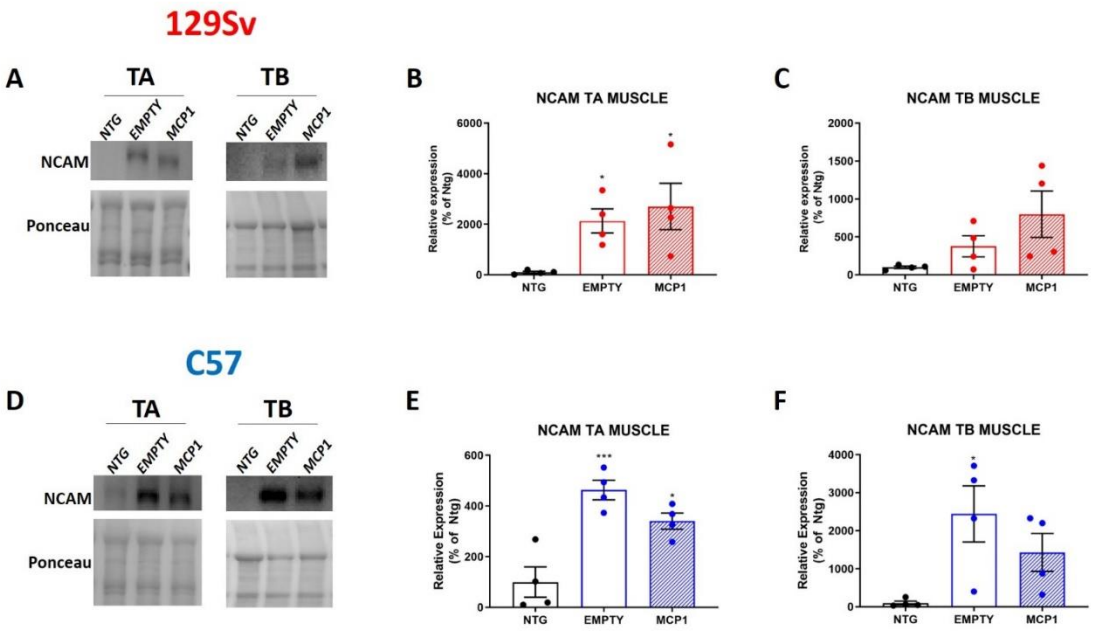

Figure 24: *Representative immunoblot images of the Neural Cell Adhesion Molecule (NCAM) in the tibialis anterior (TA) and triceps brachii (TB) muscle extracts of 129Sv SOD1^{G93A} (A) and C57 SOD1^{G93A} mice (D). Densitometric analysis indicated a significant expression of NCAM in the TA muscle of both fast (B) and slow progressing (E) ALS mice compared with their respective Ntg littermates. No difference in the NCAM expression was recorded in the TB muscle of 129Sv strain (C). Conversely, NCAM resulted significantly expressed in the TB muscle of scAAV9(empty) but not scAAV9_MCP1 -treated C57 SOD1^{G93A} mice compared with the Ntg littermates (F). Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05; ***p<0.001 Ntg Vs EMPTY or MCP1 by ANOVA with Tukey's post-analysis.*

6.6 DISCUSSION

MCP1 is a chemokine with a well know proinflammatory activity. However, recent evidence demonstrated its involvement in wound healing and regenerative processes (Deshmane et al., 2009). This protective effect comes mainly from its chemoattractant capability toward macrophages (Oishi and Manabe 2018; Papa et al., 2018; Spiller and Koh 2017). Nevertheless, several pieces of evidence depicted a pleiotropic neuroprotective role of MCP1 when expressed by neuronal cells (Locatelli et al., 2012; Papa et al., 2018; Matsubara et al., 2015; Kwon et al., 2015).

We have recently observed that MCP1 was less activated in 129Sv SOD1^{G93A} mice at both central and peripheral level compared with C57 SOD1^{G93A} mice, and this correlated with a faster and more aggressive disease progression (Nardo et al. 2013; 2016b).

Base on this evidence, we tested the effect of early induction of MCP1 on the disease progression of fast and slow progressing ALS models. Unexpectedly, the treatment differently influenced the clinical phenotype of the two strains of ALS mice. In the C57 SOD1^{G93A} mice, the MCP1 induction ameliorated the disease progression postponing the appearance of the muscle strength deficit.

Conversely, in the fast progressing mice, the chemokine induction did not have any effect on the motor onset but led to a worsening of the clinical phenotype in the late phase of the disease.

Intriguingly, although the chemokine transcription level was higher in the TB muscle of the fast progressing compared with the slow progressing mice upon the scAAV9_MCP1 injection (fold change 129Sv SOD1^{G93A}: 42.3±9.7; fold change C57 SOD1^{G93A}: 16.1±1.0), the forepaw muscle was protected from the denervation atrophy in the C57 but not in 129Sv SOD1^{G93A} mice.

Counterintuitively to the behavioural data and muscle wasting, the AChRγ transcript analysis suggested a reduced NMJs denervation in the TA muscle of 129Sv but not C57 SOD1^{G93A} mice upon MCP1 induction. Nevertheless, the histological and western blot analyses did not reveal any difference in terms of NMJs denervation in the two experimental groups either of fast or slow progressing ALS mice. The opposite result recorded between the gene expression and histological/western blot analysis might derive from a background-related difference in the turnover of the AChR subunits during the disease progression of two ALS models (Rudolf et al., 2013; 2014). However, further assessment will be necessary to clarify this discrepancy. Intriguingly, preliminary data obtained in our laboratory showed lower transcription of the adult AChRε and reduced response to the acetylcholine neurotransmitter of the TA muscle NMJs of fast progressing compared with slow progressing ALS mice, which could partly explain the opposite effect recorded in the skeletal muscles analysis of the two ALS strains upon MCP1 induction. However, the investigation of the AChR subunits rearrangement between the two SOD1^{G93A} models is part of another project of our laboratory; therefore, it has not been further examined in this context.

Altogether, these data suggest that the effect (beneficial or detrimental) deriving from the MCP1 induction is strictly dependant from the genetic background of the SOD1^{G93A} models. Understanding which factors are implicated in such controversial results might pave the way to identify potential biomarkers useful to drive a specific therapy in ALS patients.

Besides, the feeble effect of the chemokine on the NMJs innervation despite its significant upregulation in the TA muscle of both murine strains (fold change 129Sv SOD1^{G93A}: 757.1±208.5; fold change C57 SOD1^{G93A}: 542.1±82.5) might be due to the temporal and regional involvement of

hindlimbs compared to forelimbs in the pathoprogression of SOD1^{G93A} mice (Beers et al., 2011b; Capitanio et al., 2012; Clark et al., 2016; Nardo et al., 2018). Indeed, it is well known that the mSOD1 murine model is characterised by ascending paralysis, which mainly affects hindlimbs and, afterwards, the forelimbs (Gurney et al., 1994; Bruijn et al., 1997). Accordingly, it is possible that the differential biomolecular effect resulting from the chemokine induction in the hind paws muscles of the two ALS strains was no more detectable at the symptomatic stage. To confirm this hypothesis, in the next section of this Thesis, we deeper investigated the mechanism of action resulting from the scAAV9_MCP1 injection in the lower motor units of ALS mice.

RESULTS

Chapter VII

Mechanism of action of MCP1 induction in fast and slow progressing ALS mice: focus on the lower motor units

7.1 BACKGROUND and AIM

Recent observations have demonstrated the pivotal effect of MCP1-mediated phlogosis in wound healing and regenerative processes (Eming et al., 2017). Furthermore, it has been shown the beneficial involvement of this inflammatory chemokine within the main body compartments affected by ALS: MN (Locatelli et al., 2012; Conductier et al., 2010), motor axon (Deng et al., 2015; Liu et al., 2019b) and skeletal muscle (Shireman et al., 2007; Martinez et al., 2010; Lu et al., 2011a). Given the capability of the experimental protocol herein used at transducing the entire motor unit of ALS mice, as demonstrated by the specific transduction of skeletal muscles, nerves and MN soma following the single i.m. injection of scAAV9_GFP (Chapter V), this part of the project aimed at analysing the effect of MCP1 induction in the lower motor units of fast and slow progressing ALS mice, which are the first affected by the disease (Clark et al., 2016; Beers et al., 2011b; Capitanio et al., 2012; Nardo et al., 2018).

7.2 EXPERIMENTAL DESIGN

An extensive histological and biochemical/molecular analysis was done at TA muscle, sciatic nerve and lumbar spinal cord level to characterise the indirect (mediated by the recruitment of the immune cells) and direct (pleiotropic) effect of MCP1 induction on the clinical outcome of fast and slow progressing ALS mice.

7.3 FOCUS ON THE TIBIALIS ANTERIOR MUSCLE

Muscles are the first compartment affected by the disease in both patients and ALS models (Pansarasa et al., 2014). In light of the pivotal role of the immune response in muscle regeneration (Peake et al., 2017) and thus in governing the speed of disease progression of $SOD1^{G93A}$ mice (Nardo et al., 2016b; Vallarola et al., 2018), the first step of this project was the characterisation of the biomolecular alterations occurred in the TA muscles of the two ALS models following the induction of MCP1 chemokine.

7.3.1 ANALYSIS OF THE INFILTRATION OF IMMUNE CELLS FOLLOWING MCP1 INDUCTION

The acute pro-inflammatory signalling and immune cell infiltration are the initial phase of muscle response to injury, in which the recruitment of the inflammatory cells appears to be critical for successful regeneration (Kharraz et al., 2013). Among the infiltrated inflammatory cells, monocytes and macrophages are pivotal in this process (Summan et al., 2006; Chazaud 2020).

Multiple evidence correlates the protective effect of the MCP1-mediated inflammation to its chemoattractant activity toward immune cells (Oishi and Manabe 2018; Spiller and Koh 2017), particularly on monocytes/macrophages. The temporal and spatial recruitment of macrophages represents a critical step to the muscle response to injury. Indeed, it has been demonstrated that the inhibition of the accumulation of monocytes/macrophages within injured muscles strongly impairs the regenerative response (Summan et al., 2006; Lu et al., 2011a).

Accordingly, we started our investigation from the analysis of the extent of macrophages infiltration in the TA muscle of fast and slow progressing mice following scAAV9_MCP1 i.m. injection.

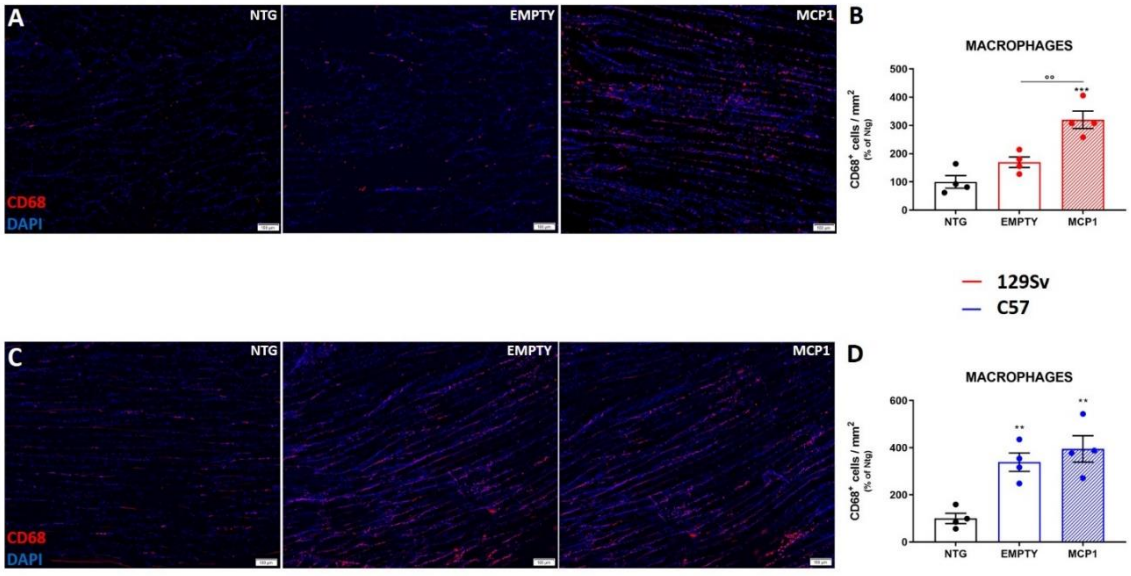

Figure 25: *(A, C) Confocal micrographs of longitudinal sections of tibialis anterior muscle of 129Sv SOD1^{G93A} (A) and C57 SOD1^{G93A} mice (C) stained with the phagocytic marker CD68 and DAPI (nucleus). Imaging analysis revealed a significant increase of macrophages recruitment in fast progressing (B) but not in slow progressing (D) ALS mice following scAAV9_MCP1 injection. Data are expressed as mean±SEM (n=4 per experimental group). **p<0.01, ****p<0.0001 Ntg vs EMPTY or MCP1; °°p<0.01 EMPTY vs MCP1 by ANOVA with Tukey's post-analysis. Scale bar= 100µm.*

Suitably to the significant upregulation of the chemokine (Fig. 21A, C), the analysis of the cells immunopositive for the CD68 phagocytic marker showed higher recruitment of macrophages within the TA muscle of fast progressing mice following MCP1 induction compared to controls (Fig. 25A, B). Conversely, in the TA muscle of C57 SOD1^{G93A} mice treated with the scAAV9(empty) we recorded significant recruitment of CD68^{+} macrophages compared to the non-transgenic littermates, immune responsivity that was not modified by the MCP1 induction (Fig. 25C, D).

Although MCP1 preferentially recruit monocytes (Rollins 1997), CCR2 is also expressed on activated T lymphocytes (Bonecchi et al., 1998; Luther and Cyster 2001), which actively participates in the regenerative mechanisms of skeletal muscles (Yang and Hu 2018; Zhang et al., 2014a; Deyhle and Hyldahl 2018). Therefore, we analysed the effect of MCP1 induction also on the recruitment of T cells within the TA muscle of the two ALS models.

As with macrophages, CD4^{+} and CD8^{+} T cells showed a heightened infiltration in the TA muscle of scAAV9_MCP1-treated 129Sv SOD1^{G93A} mice, and this occurred along with the increased levels of FoxP3 transcript, a marker of T regulatory (Treg) cells (Fig. 26A-C). Conversely, MCP1 induction did not modify the ability of slow progressing mice in also recruiting the T lymphocytes within the hind paw muscle (Fig. 26D-F).

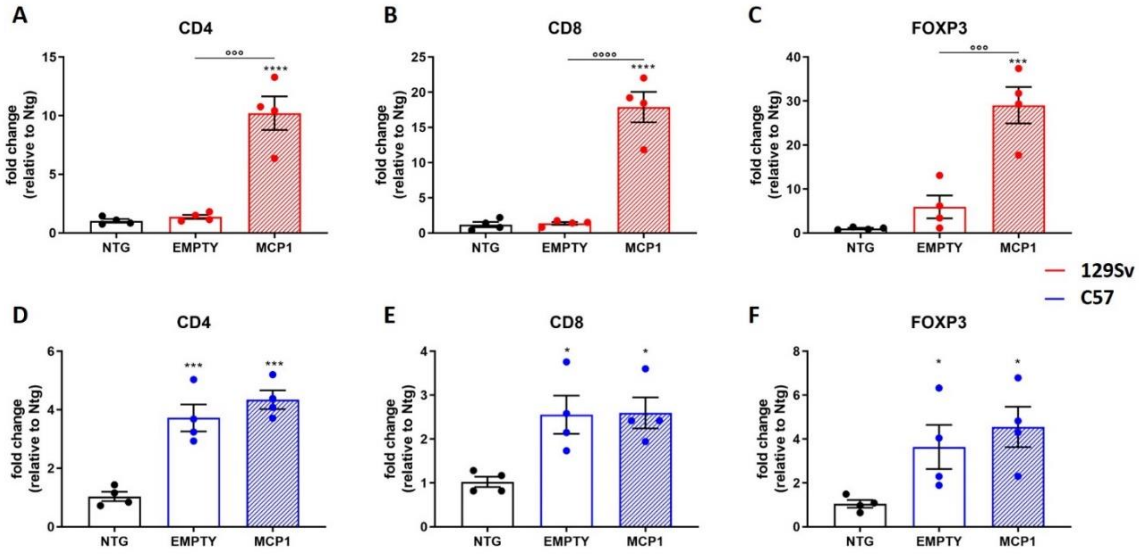

Figure 26: *Real-Time PCR analysis of CD4, CD8 and Foxp3 transcripts in the tibialis anterior muscle of 129Sv SOD1^{G93A} (A-C) and C57 SOD1^{G93A} mice (D-F) compared with their respective non-transgenic (Ntg) littermates. The scAAV9_MCP1 injection increased the recruitment of T lymphocytes in fast progressing but not in slow progressing ALS mice. Data are normalised to β-actin and expressed as mean±SEM (n=4 per experimental group). **p<0.01, ***p<0.001, ****p<0.0001 Ntg vs EMPTY or MCP1; °°°p<0.001, °°°°P<0.0001 EMPTY vs MCP1 by ANOVA with Tukey's post-analysis.*

7.3.2 CHARACTERISATION OF THE MUSCULAR INFLAMMATORY MILIEU FOLLOWING MCP1 INDUCTION

A large amount of evidence reported that the inflammation is pivotal at regulating the regenerative mechanisms of the skeletal muscle. Indeed, it participates to the activation of the prompt tissue response to damage, governing the concerted interaction of the numerous actors (e.g. resident and infiltrating immune cells, satellite cells) involved in this process (Yang and Hu 2018; Tidball 2017). However, the inflammatory milieu must be finely regulated as it represents a limiting step for the achievement of muscle regeneration and thus to return to tissue homeostasis (Howard et al., 2020; Musarò 2014; Urso 2013). Indeed, while the acute inflammation bridges from the muscle necrosis to the preparation of a strong response to injury (Rigamonti et al., 2013), its resolution is necessary to wound healing (Mann et al., 2011; Howard et al., 2020). To accomplish this regenerative mechanism within muscles, immune cells, particularly macrophages (Saclier et al., 2013b), must switch from the M1 (pro-inflammatory) to the M2 (anti-inflammatory) phenotype to establish a permissive milieu for wound healing (Rigamonti et al., 2014; Tidball et al., 2014; Arnold et al., 2007; Kharraz et al., 2013; Wang et al., 2014a).

Therefore, we analysed the inflammatory milieu within the TA muscle of fast and slow progressing $SOD1^{G93A}$ mice following MCP1 induction to correlate its polarisation (M1 or M2) to the responsiveness of tissues to ALS damage.

The data collected showed that the TA muscle of fast progressing treated mice was polarised toward a pro-inflammatory milieu, as demonstrated by the increased transcription of Tumour Necrosis Factor-alpha (TNFα) cytokine and the expression of $gp91^{PHOX}$ (heme-binding subunit of the NADPH oxidase) compared to the control group (Fig. 267-C). Conversely, upon MCP1 induction, in the TA muscle of C57 $SOD1^{G93A}$ treated mice we recorded a reduced expression of $gp91^{PHOX}$ and a trend in the downregulation of TNFα transcript compared with the scAAV9(empty) treated mice (Fig. 27D-F).

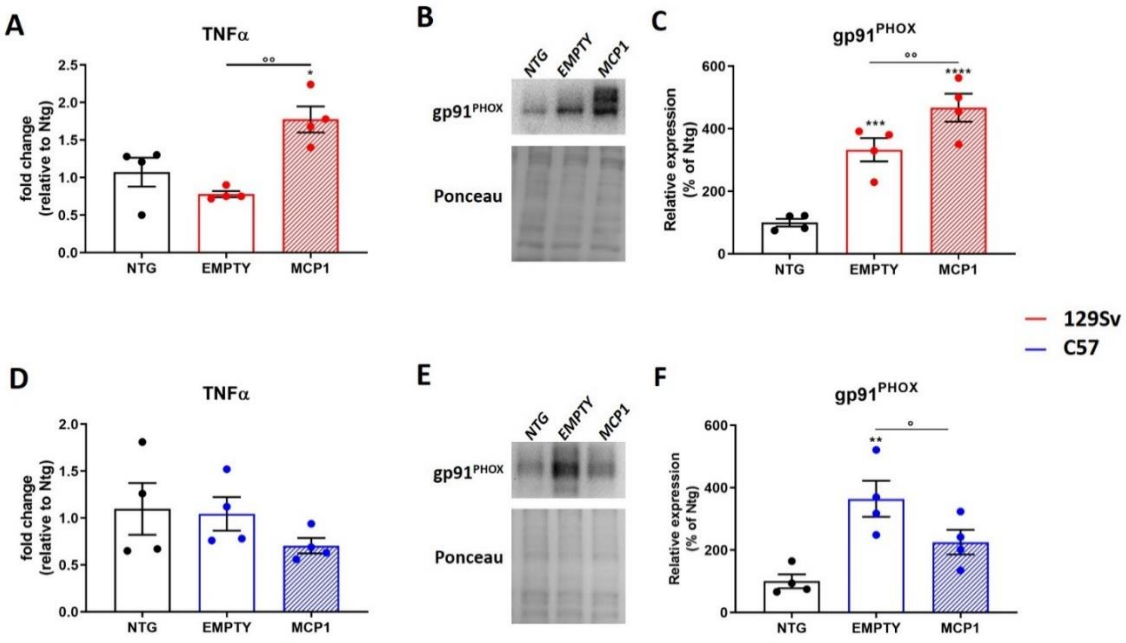

Figure 27: *Real-Time PCR analysis of TNFα transcript in the tibialis anterior muscle of 129Sv SOD1^{G93A} (A) and C57 SOD1^{G93A} mice (D) compared with their respective non-transgenic (Ntg) littermates. The scAAV9_MCP1 injection increased the transcription of TNFα in fast progressing but not slow progressing ALS mice. Data are normalised to β-actin and expressed as mean±SEM (n=4 per experimental group). Representative immunoblot images of gp91^{PHOX} in the tibialis anterior muscle extract of 129Sv SOD1^{G93A} (B) and C57 SOD1^{G93A} mice (E) compared with their respective Ntg littermates. Densitometric analysis indicated that, following scAAV9_MCP1 injection, gp91^{PHOX}expression is increased in fast progressing (C) while it was reduced in slow progressing mice (F) compared with the control group. Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05, °°p<0.01 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

However, we did not observe any significant difference in the expression of the anti-inflammatory marker Arginase 1 in the TA muscle of both fast and slow progressing treated mice compared with their respective control group of scAAV9(empty) treated animals (Fig. 28A, B, D, E).

Recent evidence highlighted the involvement of the Insulin-like Growth Factor 1 (IGF1) in orchestrating muscle regeneration through the modulation of the macrophages switching from the M1 toward the M2 phenotype (Tonkin et al., 2015; Mourkioti and Rosenthal 2005; Lu et al., 2011b). Therefore, we analysed the effect of the treatment on the transcriptional level of IGF1 in both ALS strains. Surprisingly, a significant downregulation of IGF1 transcript was recorded in both SOD1^{G93A} models upon scAAV9_MCP1 injection (Fig. 28C, F).

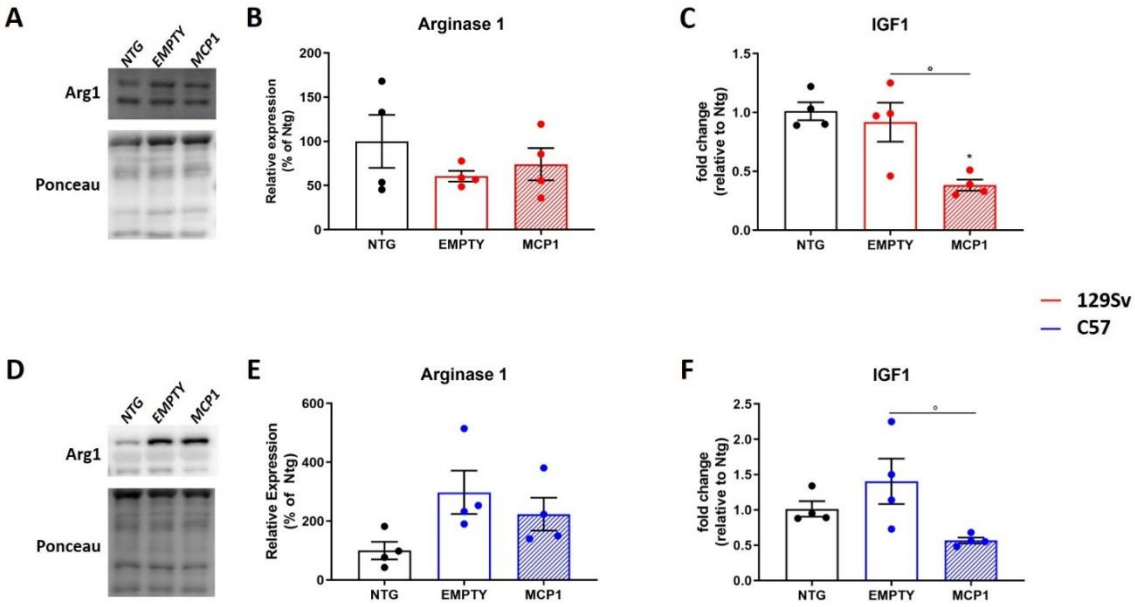

Figure 28: *Representative immunoblot images of Arginase 1 in the tibialis anterior muscle extract of 129Sv SOD1^{G93A} (A) and C57 SOD1^{G93A} mice (D) compared with their respective non-transgenic (Ntg) littermates. The densitometric analysis did not show any significant variation in the Arginase 1 expression in both strain of ALS mice upon scAAV9_MCP1 injection (B, E). Data are expressed as mean±SEM (n=4 per experimental group). Real-time PCR analysis of Insulin-like growth factor 1 (IGF1) transcript in the tibialis anterior muscle of 129Sv SOD1^{G93A} (C) and C57 SOD1^{G93A} mice (F). Following scAAV9_MCP1 injection, IGF1 transcript was significantly downregulated compared to the scAAV9(empty) treated animals in both strains of ALS mice. Data are normalised to β-actin and expressed as mean±SEM (n=4 per experimental group). *p<0.05 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

7.3.3 CHARACTERISATION OF THE EFFECT OF RECRUITED IMMUNE CELLS AND THEIR INFLAMMATORY PHENOTYPE ON THE SATELLITE CELL-MEDIATED RESPONSE FOLLOWING MCP1 INDUCTION

Numerous evidence showed that myeloid lineage cells regulate the muscle wound healing through two inductive mechanisms: a mechanism that led to the establishment of a permissive milieu for regeneration ("permissive mechanism") and an "instructive mechanism" that acts directly on myogenic progenitor cells (MPCs) (a.k.a. satellite cells) (Arnold et al., 2007; Cantini et al., 1994; Saclier et al., 2013b; Tidball 2017; Madaro et al., 2019; Dort et al., 2019; Ceafalan et al., 2018). The phagocytic M1 macrophages promote the activation and proliferation of satellite cells, while the final commitment to myocytes is supported by the M2 polarised macrophages (Kharraz et al., 2013; Oishi and Manabe 2018; Tidball et al., 2014; Tidball 2017).

Besides macrophages, the adaptive immune response is pivotal in muscle regeneration. Indeed, considerable evidence showed that T lymphocytes are instrumental in the repair/regeneration process following severe muscle damage in mice (Castiglioni et al., 2015; Madaro and Bouché 2014;

Deyhle and Hyldahl 2018). However, the role of T lymphocytes at influencing satellite cells response and/or modulating the inflammation occurring within damage site (i.e. the immunosuppressive activity of T reg cells) has yet to be elucidated (Deyhle and Hyldahl 2018).

Given the close relationship between immune cells recruitment and MPCs activation, we evaluated the extent of the satellite cell-mediated response analysing the expression of two critical myogenic transcription factors: Paired box protein 7 (Pax7), the hallmark of satellite cells stemness (Mauro 1961), and Myogenin (MyoG), a marker of early commitment and differentiation (Cornelison and Wold 1997).

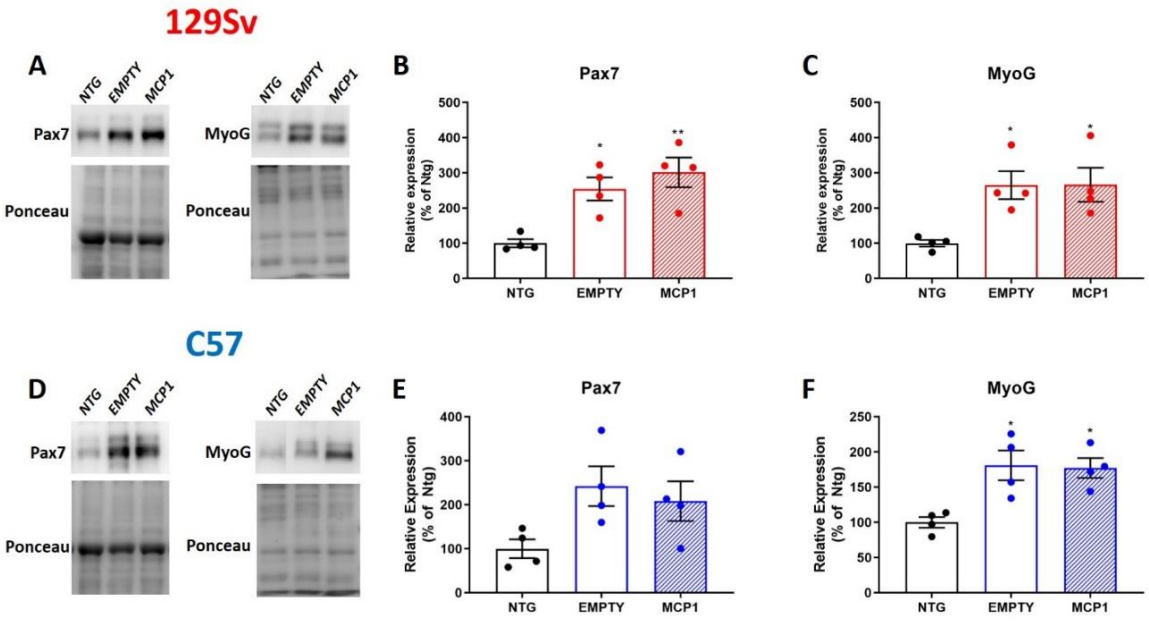

Figure 29: *Representative immunoblot images of Pax7 and Myogening (MyoG) in the tibialis anterior muscle extracts of 129Sv SOD1^{G93A} (A) and C57 SOD1^{G93A} mice (D) compared with their respective non-transgenic (Ntg) littermates. The densitometric analysis did not show any significant variation in the expression of the satellite cells transcriptional factors in both strains of ALS mice. Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01 Ntg Vs EMPTY or MCP1 by ANOVA with Tukey's post-analysis.*

Although in the TA muscle of the fast progressing scAAV9(empty) treated mice the expression level of both Pax7 and MyoG was significantly increased compared to the non-transgenic littermates, the satellite cell-mediated response was not modified by the MCP1 induction (Fig. 29A-C). Similarly, MyoG, but not Pax7, was significantly increased in the hind paw muscle of scAAV9(empty) treated mice compared with the non-transgenic littermates; however, the chemokine induction did not affect the extent of the MPCs activation also in the slow progressing mice (Fig. 29D-F). This evidence suggests that, at the symptomatic stage of the disease, the recruitment and fingerprint of immune

cells within hindlimb skeletal muscles is pointless to the regenerative response of myofibers in ALS mice.

7.4 FOCUS ON THE SCIATIC NERVE

Growing evidence suggests that distal axonal degeneration begins very early in the ALS course, long before symptom onset and MN death (Clark et al., 2016; Fischer and Glass 2007; Fischer et al., 2004). Indeed the Wallerian degeneration, a well-orchestrated morphologic and biochemical changes which involve the activation of Schwann cells (SCs) and immune cells, is a pathological feature recorded in both ALS models and patients (Deng et al., 2018; Gentile et al., 2019; Tian et al., 2016; Clark et al., 2016).

We have previously demonstrated that the extent of immune responses in the peripheral axons is pivotal in governing the speed of the disease progression of $SOD1^{G93A}$ mice (Nardo et al., 2016b). Particularly, recruited immune cells (e.g. macrophages, T cells) cooperate to remove cellular debris creating a favourable milieu for axon repair and regeneration (Gaudet et al., 2011; Jessen and Mirsky 2016; Ydens et al., 2013).

Based on the retrograde transduction of the engineered scAAV9 following the i.m. injection (Chapter V), the following step of this project was the analysis of the sciatic nerve of the two ALS models following MCP1 induction, paying specific attention to the immune-mediated response in axonal regeneration.

7.4.1 VALIDATION OF MCP1 INDUCTION AND IMMUNE CELLS INFILTRATION

The recruitment of immune cells is a fundamental step to achieve a successful axonal regeneration (Barrette et al., 2008; Liu et al., 2019b; Moalem et al., 1999; Hu and McLachlan 2002). Moreover, it has been demonstrated a pivotal role of the MCP1-mediated pathway in facilitating the recovery after an axonal injury (Zigmond and Echevarria 2019).

Accordingly, we first verified the chemokine induction and the resulting immune cells infiltration within the sciatic nerve of the two ALS models following scAAV9_MCP1 i.m. injection.

Consistently with previous evidence (Deng et al., 2018), MCP1 and CD68 transcripts resulted upregulated in both strains of scAAV9(empty) treated mice compared with their respective non-transgenic littermates (Fig. 30A, B, D, E). However, our analysis revealed increased recruitment of cytotoxic T lymphocytes in the sciatic nerve of slow progressing but not fast progressing scAAV9(empty) treated mice, as demonstrated by the different modulation of the CD8 transcript (Fig. 30C, F). Intriguingly, in the 129Sv SOD1^{G93A} mice the scAAV9_MCP1 i.m. injection increased the transcription level of the chemokine (Fig. 30A), promoting the infiltration of CD8^{+} T cells without affecting the extent of macrophage recruitment within the sciatic nerve (Fig. 30B, C). Conversely, in the slow progressing mice, the treatment did not induce a further increase in the MCP1 transcription (Fig. 30D). Noteworthy, the infiltration of macrophages, but not T cells, was significantly reduced in the sciatic nerve of C57 SOD1^{G93A} treated mice compared with the control group (Fig. 30E, F).

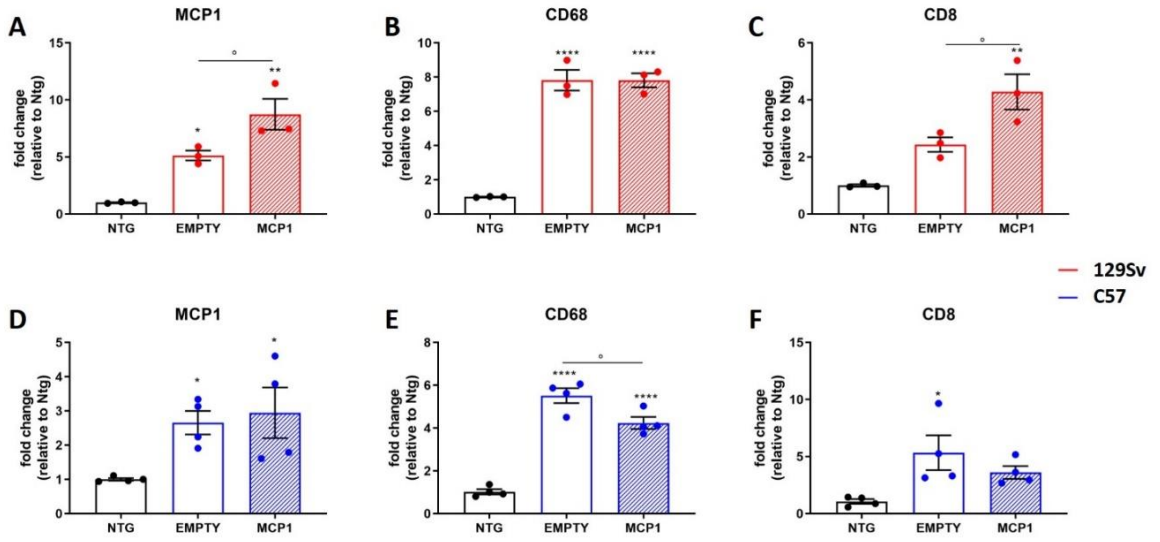

Figure 30: *Real-Time PCR analysis of MCP1, CD68 and CD8 transcripts in the sciatic nerve of 129Sv SOD1^{G93A} (A-C) and C57 SOD1^{G93A} mice (D-F) compared with their respective non-transgenic (Ntg) littermates. The scAAV9_MCP1 injection increased the transcription of MCP1 (A) and CD8 (C), but not CD68 (B) in the fast progressing mice compared with the scAAV9(empty) control. In the slow progressing mice, the treatment did not modify the transcription levels of MCP1 (D) and CD8 (F), while it downregulated the CD68 mRNA (E) compared with the scAAV9(empty) control. Data are normalised to β-actin and expressed as mean±SEM (n=3 per group 129Sv; n=4 per group, C57). *p<0.05, **p<0.01, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

It has been demonstrated that, besides damaged motor axons (Perrin et al., 2005), also injured dedifferentiated (non-myelinating) Schwann cells release proinflammatory cytokines, including MCP1 (Ydens et al., 2013), and can acquire a macrophagic phenotype following a nerve injury

(Reichert et al., 1994; Nardo et al., 2016b). Consistently we cannot exclude that the difference in the transcription levels of MCP1 and CD68 recorded in the two SOD1^{G93A} strains following the scAAAV9_MCP1 i.m. injection could derive from a different SCs-mediated response at the symptomatic stage of the disease.

7.4.2 ANALYSIS OF THE INFLAMMATORY MILIEU

The studies performed illustrated the relevance of a fine-tuned spatiotemporal expression of cytokines/chemokines in the context of peripheral nerve regeneration (Chen et al., 2015b; Dubový et al., 2013; Büttner et al., 2018). Therefore, we characterised the inflammatory milieu within the sciatic nerve of the two ALS strains following scAAV9_MCP1 injection.

The MCP1-mediated immune cells infiltration triggered the inflammation in the nerve of fast progressing mice, as demonstrated by the upregulation of TNFα (Fig. 31A). Specularly, the reduced immune cells infiltration observed in C57 SOD1^{G93A} mice following MCP1 induction, translated in a significant downregulation of TNFα compared with the scAAV9(empty) treated mice (Fig. 31B).

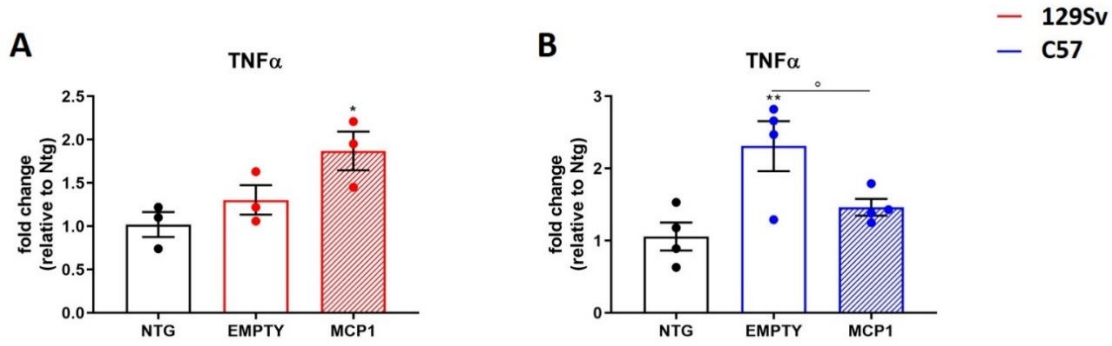

Figure 31: *Real-Time PCR analysis of TNFα transcript in the sciatic nerve of 129Sv SOD1^{G93A} (A) and C57 SOD1^{G93A} mice (B) compared with their respective non-transgenic (Ntg) littermates. The scAAV9_MCP1 injection upregulated the TNFα transcript in fast progressing mice compared with the Ntg littermates. Conversely, the treatment significantly reduced the transcription of TNFα in slow progressing mice compared with the scAAV9(empty) treated mice. Data are normalised to β-actin and expressed as mean±SEM (n=3 per group 129Sv; n=4 per group, C57). *p<0.05, **p<0.01 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

7.4.3 ANALYSIS OF NERVES AND SCHWANN CELL-MEDIATED RESPONSE

The axonal deterioration is an early event in the pathology of SOD1^{G93A} mice (Clark et al., 2016) that occurs prior than the degeneration of myelin and Schwann cells (Deng et al., 2018). Notably, it has been reported the MCP1 is pivotal at sustaining the PNS regeneration after injury (Zigmond and Echevarria 2019; Niemi et al., 2016).

Therefore, we investigated the effect of the early induction of MCP1 on the maintenance of motor axon structure and function. The expression of neurofilament (NF200), the main constituent of the axonal cytoskeleton (Lee and Shea 2014), and β-importin, a karyopherin involved in transducing damage signals from the axons of injured neurons back to the cell body (Hanz et al., 2003), were analysed in the sciatic nerve of the two strains of SOD1^{G93A} mice.

The immunoblot analysis showed that MCP1 induction did not modify the retrograde injury signalling in both strains of ALS models, as demonstrated by the unchanged expression level of β-importin compared with the respective scAAV9(empty) treated animals (Fig. 32A-D). Notably, the chemokine induction led to full maintenance of the structural integrity of motor axons in slow progressing mice, given the unchanged expression of NF200 in scAAV9_MCP1 treated mice compared with non-transgenic littermates (Fig. 32G, H). Conversely, in fast progressing mice, which are characterised by a higher impairment of sciatic nerve compared with C57 SOD1^{G93A} mice (Nardo et al., 2016b), the early induction of MCP1 was not sufficient to significantly protect the axonal structure from ALS degeneration (Fig. 32E, F).

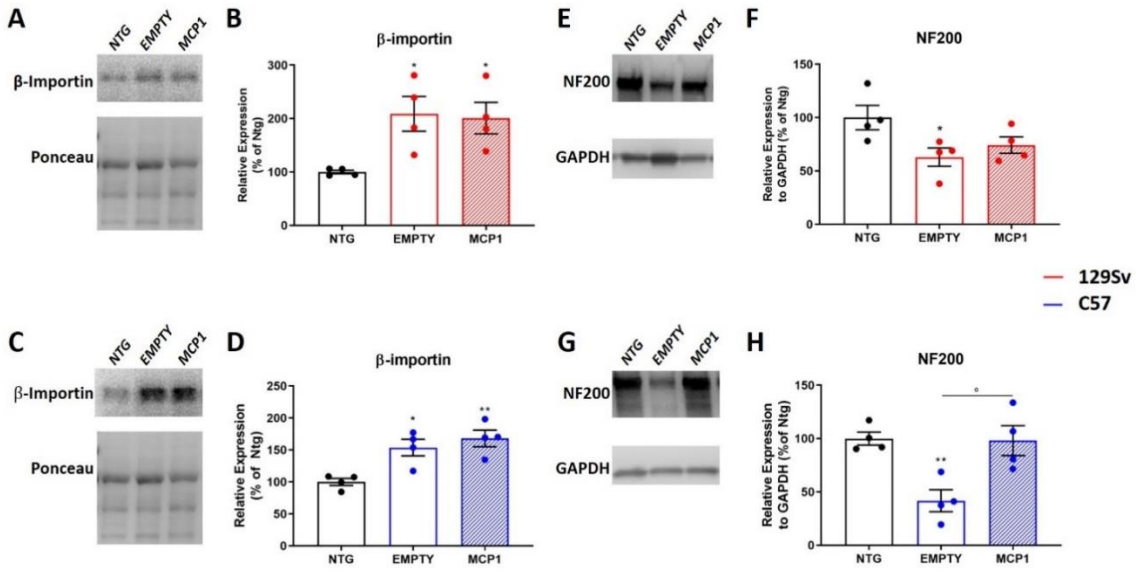

Figure 32: *Representative immunoblot images of β-importin and neurofilament (NF200) in the sciatic nerve extracts of 129Sv SOD1^{G93A} (A, E) and C57 SOD1^{G93A} mice (C, G) compared with their respective non-transgenic (Ntg) littermates. The densitometric analysis did not show any significant variation in the expression of β-importin in both strains of ALS mice following the treatment (B, D). The scAAV9_MCP1 injection significantly preserved the cytoarchitecture of motor axons is slow progressing (H), but not fast progressing mice (F). Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

Following an acute nerve injury, the Schwann cells associated to the damaged axons dedifferentiate, proliferate and, in concerted action with infiltrated immune cells, clear the apoptotic debris paving the way for a permissive milieu for regeneration (Gaudet et al., 2011; Jessen and Mirsky 2019). Indeed, it has been demonstrated that the first response of SCs to damage consists in the reversing of their molecular expression toward a progenitor-like state activating genes usually found on immature cells, such as $p75^{NTR}$ (Jessen and Mirsky 2008). However, it has been recently reported that ALS mice failed at activating $p75^{NTR}$ after damage (Deng et al., 2018), suggesting a severe impairment in the activation of the SCs-mediated response and remyelination upon an injury (Tomita et al., 2007; Song et al., 2006). According to the preservation of the cytoarchitecture of the nerve, $p75^{NTR}$ was upregulated in slow progressing but not fast progressing mice following MCP1 induction (Fig. 33A-D). Suitably, our analysis showed that C57 $SOD1^{G93A}$ treated mice had unchanged myelin basic protein (MBP) levels compared to non-transgenic littermates, while fast progressing mice showed a remarked downregulation both in the presence and absence of MCP1 induction (Fig. 33E-H).

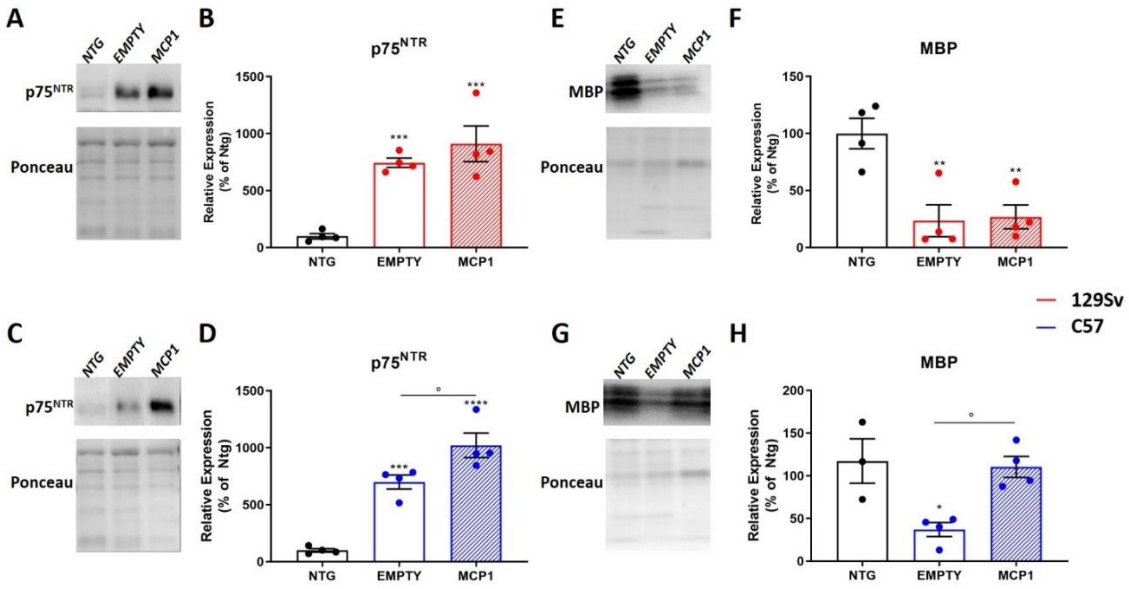

Figure 33: *Representative immunoblot images of $p75^{NTR}$ and Myelin Basic Protein (MBP) in the sciatic nerve extracts of 129Sv $SOD1^{G93A}$ (A, E) and C57 $SOD1^{G93A}$ mice (C, G) compared with their respective non-transgenic (Ntg) littermates. The scAAV9_MCP1 injection in fast progressing mice did non modify the expression of $p75^{NTR}$ (B) and MBP (F) compared with the scAAV9(empty) treated mice. Conversely, the densitometric analysis indicated a significant increase in the expression of $p75^{NTR}$ (D) and MBP (H) in slow progressing treated mice compared with the control group (empty vector). Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

7.5 FOCUS ON THE LUMBAR SPINAL CORD

The analysis of GFP distribution showed that the scAAV9 spreads retrogradely from the injected skeletal muscle alongside the motor unit of ALS mice, finally transducing the motor neuron soma (Chapter V).

Although the physiologic or pathologic role of MCP1 in the CNS is well known (Conductier et al., 2010; Madrigal and Caso 2014; Semple et al., 2010b), the evidence relating to its neuroprotective capability are narrowed. Most investigations have correlated the beneficial effect of the chemokine to its ability at modulating the blood-brain-barrier permeability (Dzenko et al., 2005) and the polarisation of the recruited macrophages (Matsubara et al., 2015). However, it has been demonstrated that hematogenous monocytes do not infiltrate the CNS of $SOD1^{G93A}$ mice (Chiu et al. 2009; 2013; Kunis et al., 2015; Chiot et al., 2020).

We previously showed a significant upregulation of MCP1 in laser captured MNs from slow progressing compared to fast progressing ALS mice (Nardo et al., 2013), and recent studies demonstrated the beneficial and direct effect of MCP1 on neuron somata and motor axons (Locatelli et al., 2012; Papa et al., 2018). These data hinted at a pleiotropic and neuronal-specific MCP1 effect in the CNS. Therefore, we analysed whether the specific induction of MCP1 within MN soma was able to protect neurons from degeneration and exert a modulatory influence on the inflammatory milieu within the lumbar spinal cord.

7.5.1 VALIDATION OF MCP1 INDUCTION AND ANALYSIS OF ITS EFFECT ON LUMBAR MOTOR NEURONS SURVIVAL

We first verified the chemokine induction in the lumbar tract of the spinal cord of the two ALS models following scAAV9_MCP1 i.m. injection.

As previously reported (Henkel et al., 2006), at the symptomatic stage of the disease, MCP1 was significantly upregulated in the spinal cord of $SOD1^{G93A}$ mice compared with the non-transgenic littermates. However, the scAAV9_MCP1 injection did not increase the chemokine transcription either in fast progressing or in slow progressing ALS mice compared to the scAAV9(empty) treated animals (Fig. 34A, B). This result suggests that in a full-blown stage of the disease, the strong

expression of MCP1 by mSOD1 microglia (Sargsyan et al., 2009; Butovsky et al., 2012) might overlap and mask the neuronal scAAV9-mediated overexpression of the chemokine.

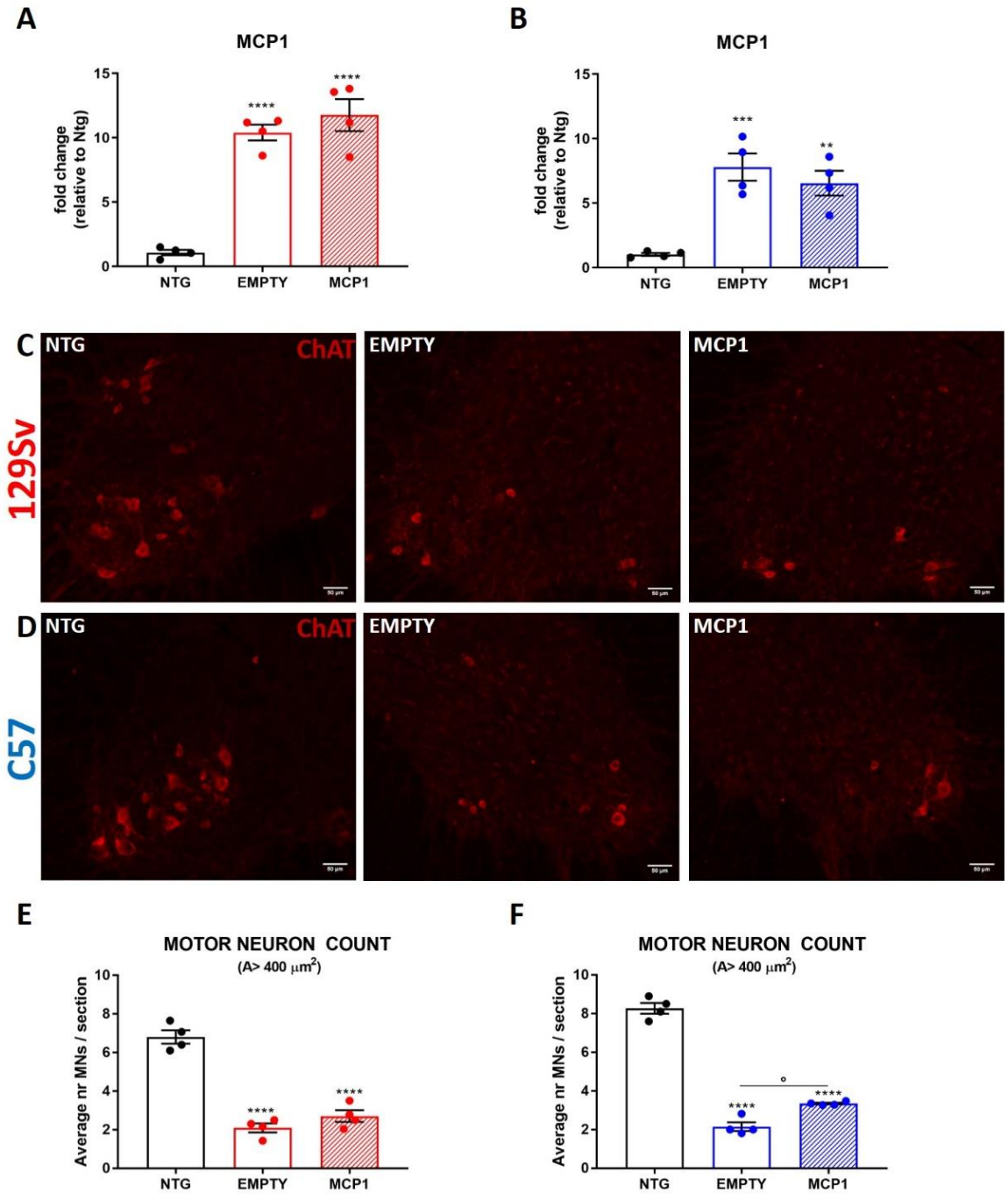

Figure 34: *Real-Time PCR analysis of MCP1 transcript in the lumbar spinal cord of 129Sv SOD1^{G93A} (A) and C57 SOD1^{G93A} mice (B) compared with their respective non-transgenic (Ntg) littermates. The scAAV9_MCP1 injection did not modify the chemokine transcription level in the CNS of both strains of ALS mice. Data are normalised to β-actin and expressed as mean±SEM (n=4 per group). Confocal micrographs of coronal sections of the lumbar spinal cord of 129Sv (C) and C57 mice (D) stained with Choline Acetyltransferase (ChAT). Scale bar= 50μm. The scAAV9_MCP1 injection reduced the motor neuron loss in slow progressing (F) but not in fast progressing mice (E) compared with the scAAV9(empty) treated mice. Data are expressed as mean±SEM (n=4 per group). **p<0.01, ***p<0.001, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

Nevertheless, the analysis of the α-MNs (Area > 400μm^2), which are the main target of the disease (Conradi and Ronnevi 1993; Lalancette-Hebert et al., 2016), showed that the treatment significantly reduced the neurodegenerative phenomenon in the slow progressing SOD1^{G93A} mice (MN nr: 2.16±0.22, EMPTY; 3.36±0.04, MCP1) (Fig. 34D, F). Conversely, the scAAV9_MCP1 injection was ineffective to counteract the MNs loss in the lumbar spinal cord of fast progressing ALS mice (MN nr: 2.09±0.23, EMPTY; 2.70±0.30, MCP1) (Fig. 34C, E).

7.5.2 ANALYSIS OF GLIA CELLS ACTIVATION AND INFLAMMATORY MILIEU FOLLOWING MCP1 INDUCTION

Neuroinflammation is pivotal in ALS pathogenesis, actively contributing to the disease progression (Zhao et al., 2013). Indeed, ALS is considered a non-cell autonomous disease, in which other cell types besides MNs are fervently involved in the pathogenic cascade (Chiot et al., 2019; Thonhoff et al., 2018). In this context, the detrimental contribution of reactive astroglia to the disease was by now established (Boillée et al., 2006a; Ilieva et al., 2009; Valori 2013). To gain further insights on the effect of the MCP1 induction in the CNS of SOD1^{G93A} mice, we analysed the degree of activation of astrocytes (GFAP) and the proliferation of microglial cells (Iba1) in the lumbar spinal cord.

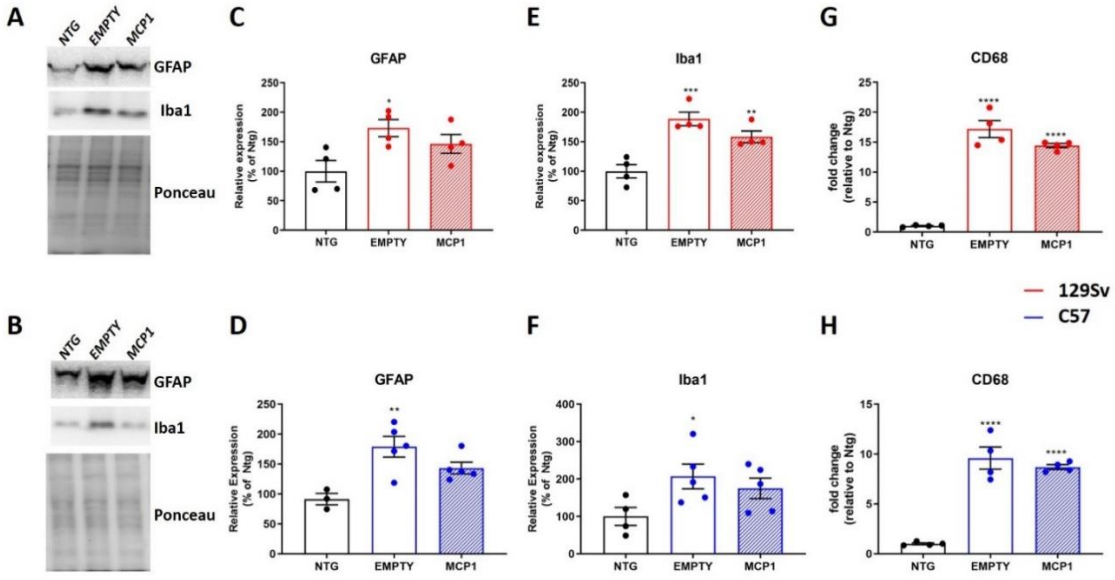

Figure 35: *Representative immunoblot images of glial fibrillary acidic protein (GFAP) and ionised calcium-binding adapter molecule 1 (Iba1) in the spinal cord extracts of 129Sv SOD1^{G93A} (A) and C57 SOD1^{G93A} mice (B) compared with their respective non-transgenic (Ntg) littermates. The scAAV9_MCP1 injection attenuated the astrogliosis in both fast (C, E) and slow (D, F) progressing ALS mice compared with the control group (empty). Data are expressed as mean±SEM (n=4 per experimental group). Real-time PCR analysis of CD68 transcript in the lumbar spinal cord of 129Sv SOD1^{G93A} (G) and C57 SOD1^{G93A} mice (H) compared with their respective non-transgenic (Ntg) littermates. The scAAV9_MCP1 injection did not*

*modify the phagocytic activity of microglia in both strains of ALS mice. Data are normalised to β-actin and expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Ntg Vs EMPTY or MCP1 by ANOVA with Tukey's post-analysis.*

As shown in Figure 35, GFAP was less expressed in the lumbar spinal cord of both ALS models following MCP1 induction. However, the treatment was able neither to prevent nor to significantly decrease the astrocytosis compared with the scAAV9(empty) treated mice (Fig. 35A, D). Likewise, the proliferation of microglia was reduced in the slow progressing mice upon MCP1 induction, although not significantly compared with the scAAV9(empty) treated group (Fig. 35F). In the fast progressing mice, we recorded a higher expression of Iba1 in both experimental groups of SOD1^{G93A} mice compared with the non-transgenic littermates, albeit lower upon MCP1 induction (Fig. 35E). Despite these differences, no variations were found in the phagocytic activity of microglia of both ALS models, as assessed by the analysis of the CD68 transcript (Fig. 35G, H).

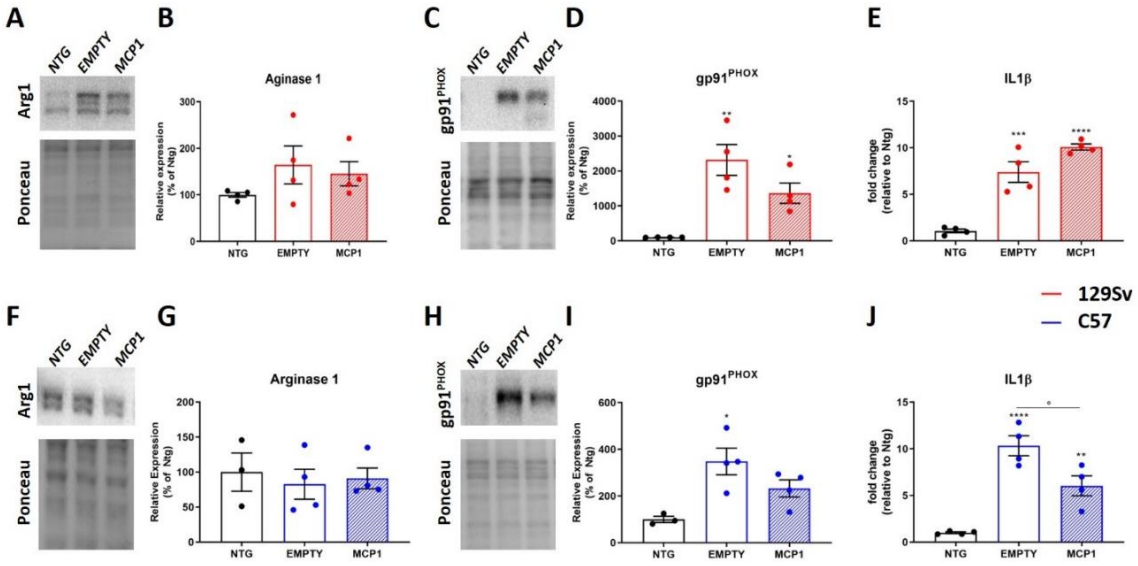

Figure 36: *Representative immunoblot images of Arginase1 and gp91^{PHOX} spinal cord extracts of 129Sv SOD1^{G93A} (A, C) and C57 SOD1^{G93A} mice (F, H) compared with their respective non-transgenic (Ntg) littermates. The densitometric analysis did not show any modification in the Arginase1 expression (B, G), while gp91^{PHOX} is slightly reduced in both strains of ALS mice upon scAAV9_MCP1 injection (D, I). Data are expressed as mean±SEM (n=4 per experimental group). Real-time PCR analysis of interleukin 1β transcript in the lumbar spinal cord of 129Sv SOD1^{G93A} (E) and C57 SOD1^{G93A} mice (J) compared with their respective non-transgenic (Ntg) littermates. Data are normalised to β-actin and expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

The activated state of microglia is an oversimplification of the range of the polarisation states (M1/M2) and its activity (toxic/protective) on MNs (Geloso et al., 2017). However, microglia fingerprint is highly dependant from the neighbouring inflammatory environment (Hanisch 2002).

Therefore, we characterised the inflammatory milieu within the lumbar spinal cord of the two ALS models.

Notwithstanding the expression of the anti-inflammatory marker Arginase 1 in $SOD1^{G93A}$ mice was not influenced by the treatment (Fig. 36A, B, F, G), we recorded reduced oxidative stress upon MCP1 induction. Indeed, the immunoblot analysis showed a strong expression of $gp91^{PHOX}$ in both strains of scAAV9(empty) treated mice compared with the respective non-transgenic littermates. However, following the chemokine induction, ALS mice showed a reduction trend in the $gp91^{PHOX}$ expression compared with the control groups (Fig. 36C, D, H, I).

Besides, following MCP1 induction, we found a significant downregulation of the IL1β proinflammatory cytokine in the spinal cord of C57 but not 129Sv $SOD1^{G93A}$ mice (Fig. 36E, J).

7.6 DISCUSSION

Although the primary hallmark is the MN death, ALS is a non-cell autonomous disorder with other cell types actively contributing to the disease including microglia, astrocytes and immune cells (Chiot et al., 2019; Thonhoff et al., 2018). Moreover, ALS encompasses distant biological systems (muscle, nerves, spinal cord, brain), and makes it even more challenging to identify a proper therapeutic target (Silani et al., 2017).

Mounting evidence highlighted the different contribution of the inflammatory response in the CNS compared with the periphery (i.e. nerves and muscles) in ALS (Dibaj et al., 2011; Chiu et al., 2009). Indeed, the aberrant glial cells activation, T cells infiltration and the resulting release of pro-inflammatory factors drive the neurodegenerative phenomenon (Chiot et al., 2019; Thonhoff et al., 2018). Conversely, the successful axonal and muscle regeneration depends on the coordinated efforts of immune cells that, besides removing cellular debris, release factors that support the wound healing (Deng et al., 2018; Van Dyke et al., 2016; Gaudet et al., 2011; Sass et al., 2018). However, the contribution of the immune response to ALS progression is still elusive (McCombe and Henderson 2011; Iyer et al., 2018).

MCP1 is usually released to exert a potent chemotactic activity by binding the C-C chemokine receptor type 2 (CCR2) on target cells, such as macrophages, microglia and T cells. The MCP1-CCR2

pathway leads to pathological microgliosis and inflammation in chronic disorders (Semple et al., 2010b), including ALS (Henkel et al., 2006; Martínez et al., 2020). Nonetheless, considerable evidence depicted MCP1 as a neuroprotective factor involved in modulating the blood-brain-barrier permeability (Dzenko et al., 2005) and promoting neurogenesis (Chintawar et al., 2009; Liu et al., 2007) and axonal elongation and outgrowth (Locatelli et al., 2012; Papa et al., 2018).

The chemotactic activity of MCP1 toward leucocytes (monocytes and T lymphocytes) is crucial in triggering wound healing and regenerative processes (Ridiandries et al., 2018). Indeed, macrophages and T cells infiltration within the damaged area is a critical step for nerves (Liu et al., 2019b; Chen et al., 2015b; Zigmond and Echevarria 2019) and muscles (Shireman et al., 2007; Martinez et al., 2010; Dort et al., 2019; Zhang et al., 2014a; Deyhle and Hyldahl 2018; Yang and Hu 2018) regeneration, which are the first body compartments affected by ALS (Moloney et al., 2014).

In the present study, we showed that the early induction of MCP1 in the motor units of fast and slow progressing SOD1^{G93A} mice induced opposite clinical outcomes, which are related to the strain-specific differences at activating the immune response and thus promoting the tissue reaction to damage.

At the symptomatic stage, the intramuscular injection of scAAV9_MCP1 exacerbates inflammation in 129Sv SOD1^{G93A} mice, while it is ineffective in counteracting the skeletal muscle degeneration in C57 SOD1^{G93A} mice

MCP1 has a pivotal role in the recruitment and modulation of immune cells thus governing the wound healing of skeletal muscle (Contreras-Shannon et al., 2007; Shireman et al., 2007; Lu et al., 2011).

Here, we reported that MCP1 transcript level is equally and remarkably increased in the skeletal muscles of both ALS models also several weeks after the single scAAV9_MCP1 i.m. injection. Nevertheless, this resulted in a differential response of the skeletal muscles of the fast compared with the slow progressing ALS mice to the treatment. 129Sv SOD1^{G93A} mice showed a heightened infiltration of macrophages and T lymphocytes in the TA muscles, while C57 SOD1^{G93A} are refractory to further increase immune cell recruitment, despite the significant increase of MCP1 levels.

Intriguingly, the increased immune cells infiltration in 129Sv SOD1^{G93A} mice exacerbated the muscle force impairment. Conversely, the C57 SOD1^{G93A} mice showed an amelioration of the motor performance compared to controls. This result suggests that triggering the immune cell infiltration in the skeletal muscles late in the disease is detrimental in mSOD1 mice.

We have previously demonstrated that 129Sv SOD1^{G93A} mice are less prone than C57 SOD1^{G93A} mice at activating an effective peripheral immune response through macrophage and T cells recruitment in the PNS and skeletal muscles (Vallarola et al., 2018; Nardo et al., 2016b). Here, we confirmed this evidence showing that, at the symptomatic stage, scAAV9(empty) and scAAV9_MCP1 treated C57 SOD1^{G93A} mice exhibit a similar extent in immune cells infiltration. Conversely, an exogenous boosting of MCP1 is necessary to promote the leucocytes infiltration in the skeletal muscle of 129Sv SOD1^{G93A} mice, suggesting a strain-related modulation of the inflammatory response following an injury.

The reduced ability at recruiting inflammatory cells has been already reported in 129Sv compared with C57 genetic background (White et al., 2002; Hoover-Plow et al., 2008). Furthermore, 129Sv mice have an intrinsic propensity in exacerbating the proinflammatory status following the injection of the lipopolysaccharides (LPS) endotoxin (Piirsalu et al., 2020). This evidence suggests a dysregulated immune system in 129Sv mice that led to an impairment in the effective management of the immune response once activated.

The sustained inflammation impairs the skeletal muscle regeneration inasmuch the establishment of a permissive environment is necessary to guarantee and achieve functional results (Yang and Hu 2018; Howard et al., 2020; Musarò 2014). Our data showed that the immune cells infiltrated in the TA muscle of the fast progressing ALS mice exhibited a pro-inflammatory fingerprint and failed to switch towards the M2 phenotype, as assessed by the reduced transcription of IGF1, which is pivotal in macrophage polarisation and inflammation resolution upon muscle injury (Tonkin et al., 2015; Tidball and Welc 2015; Pelosi et al., 2007). In parallel, the increased infiltration of T reg cells in 129Sv SOD1^{G93A} mice upon MCP1 induction might be an attempt to decrease the established phlogosis through the interaction with the innate immune cells (Li et al., 2018; Schiaffino et al.,

2017). However, it has been demonstrated that the phenotypic abilities of T reg cells to suppress inflammation is reduced in mSOD1 mice at the advanced disease stage (Beers et al., 2011a), which might explain the remarked expression of TNFα and gp91^{PHOX} recorded in the TA muscle of 129Sv SOD1^{G93A} treated mice despite the higher FoxP3 transcription compared with the control group.

Unlike the fast progressing mice, the scAAV9_MCP1 injection did not affect the extent of the immune cells recruitment in the skeletal muscle of C57 SOD1^{G93A} mice. However, TNFα, gp91^{PHOX} and IGF1 were downregulated in the scAAV9_MCP1 treated mice compared with controls, indicating the mitigation of inflammation thanks to the phenotypic switch of macrophages from the M1 to the M2 fingerprint. Noteworthy, this effect was not associated with the immunomodulatory intervention of the T reg cells, suggesting a better capability of C57 SOD1^{G93A} mice in governing a functional immune response.

The downregulated transcription of IGF1 recorded in both ALS models may also suggest a failure in the proliferation or differentiation of the satellite cells at the symptomatic stage of the disease (Manzano et al., 2011; 2013). Indeed, IGF1 is a potent myogenic factor (Tonkin et al., 2015; Dobrowolny et al., 2005), and its downregulation might correlate with the substantial impairment and damage of the hindlimbs of SOD1^{G93A} mice at the advanced stage of the disease.

In conclusion, the MCP1-enhanced immune cells recruitment profoundly altered the muscular environment and the muscle strength of fast progressing mice, suggesting a detrimental role of the immune activation and inflammation in these mice possibly due to the impairment of the 129Sv strain in coordinating a functional and protective immune response (Piirsalu et al., 2020; White et al., 2002; Hoover-Plow et al., 2008). Conversely, the data on C57 SOD1^{G93A} mice suggested that, once the disease progresses to the advanced stage, the immune-mediated response, fostered by MCP1, is worthless. Nevertheless, given that the MCP1 induction resulted in the postponement of the motor impairment in the slow progressing ALS mice, it is conceivable that a protective immune response might have occurred earlier in the disease. Indeed, immune cell infiltration and skeletal muscle impairment are early events in the pathogenic cascade of ALS (Pansarasa et al., 2014).

Therefore, only the last glimpse of the protective effect mediated by the MCP1 induction was detectable in the hid paws of C57 SOD1^{G93A} mice at the symptomatic disease stage.

The intramuscular injection of scAAV9_MCP1 preserves motor axons of C57 SOD1^{G93A} mice but not 129Sv SOD1^{G93A} mice

As muscles, axons are affected early in the ALS pathogenic cascade (Clark et al. 2016; Gentile et al. 2019).

We previously showed that the peripheral immune cell response during the first disease stages has a beneficial role in promoting Schwann cells proliferation and axonal regeneration. Conversely, at the disease onset, the fast progressing ALS mice failed to activate a consistent peripheral immune response in the sciatic nerves, and this resulted in earlier denervation of skeletal muscles (Nardo et al., 2016b). Nevertheless, here we show that, at the symptomatic stage of the disease, the 129Sv SOD1^{G93A} mice are able to activate the MCP1-mediated axis as well as recruit macrophages and T lymphocytes within the sciatic nerve. However, this tardive activation translates in a delayed immune response to injury, which, as demonstrated in aged mice (Büttner et al., 2018), proves to be ineffective in promoting axonal regeneration, remyelination and the maintenance of the nerve cytoarchitecture. Moreover, the further enhancement of the chemokine through the injection of scAAV9_MCP1 did not improve this process but instead promoted the recruitment of immune cells ($CD8^+$ T lymphocytes) with an inflammatory phenotype, which, as demonstrated in the Experimental Autoimmune Neuritis model (Ydens et al., 2013), hampers the regeneration. This evidence suggests a detrimental role of a dysregulated immune response and inflammation in the PNS of ALS mice.

Conversely, the slow progressing mice had an earlier and robust peripheral immune response since the disease onset, and this resulted in the preservation of myelin sheets and motor axon neurofilaments compared with fast progressing mice (Nardo et al., 2016b). Nevertheless, the scenario at the symptomatic stage of the disease depicted an intense inflammation which, as observed in 129Sv SOD1^{G93A} treated mice and old injured mice (Büttner et al., 2018), is ineffective to counteract the PNS degeneration as confirmed by the significant impairment of the locomotor

ability at 20 weeks. However, we can surmise that the scAAV9-mediated MCP1 induction in the sciatic nerves of C57 SOD1^{G93A} mice since the early stage of the disease has further enhanced the response to stress boosting the recruitment of immune cells in the first stage of the disease, as demonstrated by their switching toward a pro-regenerative anti-inflammatory phenotype. Thus, although at the symptomatic stage chemokine levels are similar in the sciatic nerves of scAAV9_MCP1 and scAAV9(empty) treated mice, the early MCP1 boosting reduced the tissue phlogosis and maintained the axon-cytoarchitecture and myelin wrapping around motor axons of slow progressing mice.

The intramuscular injection of the scAAV9_MCP1 exerts a protective effect in the CNS of SOD1^{G93A}mice

Several pieces of evidence depicted MCP1 as a neuroprotective factor in the CNS (Dzenko et al., 2005; Chintawar et al., 2009; Liu et al., 2007; Locatelli et al., 2012; Papa et al., 2018; Matsubara et al., 2015). Most of them correlated the beneficial effect of the chemokine to its ability in modulating the blood-brain-barrier permeability (Dzenko et al., 2005) and positively influencing the polarisation of infiltrated macrophages (Matsubara et al., 2015; Kwon et al., 2015). However, limited evidence demonstrated the direct beneficial effect of MCP1 in neuron perikarya and axons (Locatelli et al., 2012; Papa et al., 2018).

The analysis performed showed that the treatment was able to attenuate the activation of the glia cells reducing the oxidative stress within the spinal cord of ALS models. Notably, the neuronal induction of MCP1 delayed the lumbar MN loss in C57 SOD1^{G93A} mice by reducing inflammation. Conversely, this effect was absent in 129Sv SOD1^{G93A} mice, suggesting that glial cells of fast progressing ALS mice are more refractory to the modulative capability of neuronal MCP1. Further analysis will be necessary to assess the influence of MCP1 on non-neuronal neighbourhoods within the spinal cord of ALS mice.

In conclusion, the data collected indicate that the clinical response of SOD1^{G93A} mice to the induction of MCP1 within the neuromuscular system may be beneficial, detrimental or ineffective depending on the mouse genetic background, its immune-related capability and the time of

intervention. This evidence may explain the failure of the immune-modulatory/-suppressive treatments so far tested in clinical trials in which the cohorts were composed by patients genetically heterogeneous and in the full-blown stage of the disease (Wosiski-Kuhn et al., 2019; McCombe and Henderson 2011).

The data herein collected also reveal that the immune response and inflammation play a dual opposite role in governing the speed of ALS progression depending on the extent of the neuromuscular system damage. Thus, we hypothesise that at the onset of symptoms, when the damage is limited, the activation of immune response is pivotal to prevent further damage and sustain the effective regeneration of axons and skeletal muscles. On the contrary, at the later stage of the disease, this phenomenon should be restrained to avoid the detrimental effect of the inflammatory milieu, which accelerates the speed of ALS progression as demonstrated in 129Sv SOD1^{G93A} mice. To further strengthen this hypothesis, in the next chapter of this Thesis the mechanisms through which the induction of MCP1 significantly delays the motor deficit in slow progressing ALS mice will be examined at the early stage of the disease.

RESULTS

Chapter VIII

Analysis of the effect of MCP1 induction in slow progressing ALS mice at earlier time point: look back at 14 weeks

8.1 BACKGROUND and AIM

The degeneration of the peripheral compartment is an early event in ALS pathogenic cascade that occurs before the clinical manifestation of the disease (Clark et al., 2016; Azzouz et al., 1997; Fischer et al., 2004). However, the evidence describing the involvement of the immune response in governing the degeneration/regeneration or the stream of these mechanisms in the peripheral compartment during ALS progression is narrow. Besides, current knowledge on acutely damaged nerves and muscles indicates that immune cell recruitment is as an early event occurring a few hours after injury (Gaudet et al., 2011; Yang and Hu 2018). This evidence makes hard to define parallelism with the chronic and progressive degeneration occurring in ALS.

Suitably to the pivotal role of a functional and temporally appropriate immune response in promoting the wound healing following an injury, the data herein obtained in fast and slow progressing ALS mice at the symptomatic stage showed that the dysregulated response of SOD1^{G93A} mice is worthless to counteract the degeneration of the peripheral compartment. Indeed, we recorded a full-blown alteration of the TA muscle and sciatic nerve in both ALS strains at the advanced stage of the disease.

Besides, we observed that the induction of MCP1 exacerbated the (background-related) impaired immune response in the fast progressing ALS mice, worsening the clinical phenotype. Conversely, the scAAV9_MCP1 injection in C57 SOD1^{G93A} mice improved the motor ability since the early stage of the disease, postponing the disease onset. Therefore, we hypothesise that the data collected at the symptomatic phase of the disease could represent the last glimpse of the protective effect of the induction of MCP1 in slow progressing ALS mice. Hence, the next section of this thesis seeks to address the early regenerative mechanisms underlying MCP1 induction activated by slow progressing mice within the peripheral compartment. Moreover, we investigated the ability of the specific overexpression of MCP1 within MNs at modulating the neuroinflammatory phenomenon in ALS mice.

8.2 EXPERIMENTAL DESIGN

Using the experimental protocol described in section 6.2, C57 SOD1^{G93A} mice (n=8) underwent a single bilateral i.m. injection of 2.18x10^{10}vg/µL scAAV9_MCP1 in both hindlimbs (TA, GCM and GM) and forelimbs (TB) muscles (10µL per muscle) at 8 weeks of age. An empty vector was used for the treatment of the control group (n=8).

Mice were sacrificed six weeks after the i.m. injection (i.e. ~2 weeks after the virus reaches its maximum transduction efficiency (Benkhelifa-Ziyyat et al., 2013)) to analyse the effect of the MCP1 induction when the skeletal muscle of ALS mice exhibits the first signs of the disease. Therefore, an extensive histological and biochemical/molecular analysis was performed at the TA muscle, sciatic nerve and lumbar spinal cord level in 14 weeks old mice.

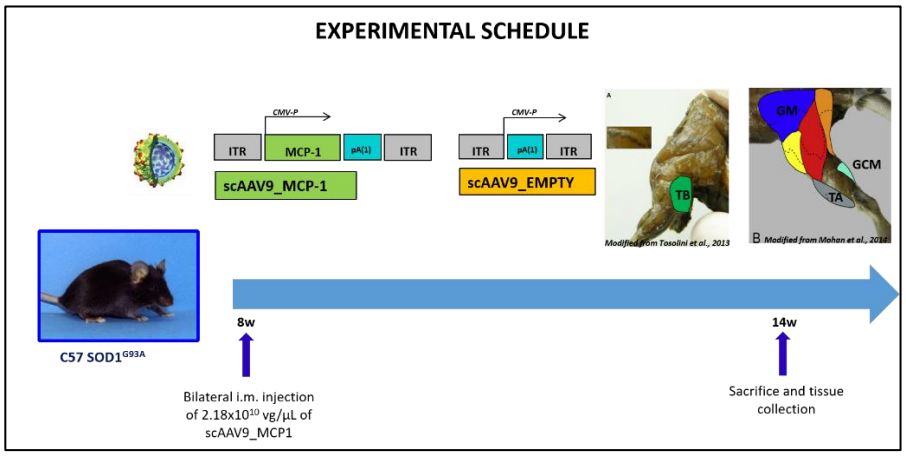

Figure 37: *Experimental schedule of i.m. injection of scAAV9_MCP1 slow progressing SOD1^{G93A} mice.*

8.3 ANALYSIS OF THE EARLY EFFECT OF MCP1 INDUCTION IN THE TIBIALIS ANTERIOR MUSCLE

8.3.1 ANALYSIS OF THE DENERVATION ATROPHY OF THE TIBIALIS ANTERIOR MUSCLE

According to the early involvement of the muscular compartment in ALS mice (Kalmar et al., 2012; Azzouz et al., 1997; Hegedus et al., 2007; Clark et al., 2016), at 14 weeks, the TA muscle of ALS mice is significantly affected by the disease. Indeed, as shown in Figure 38, our analysis recorded a reduction of 38.8±2.6% of the muscle mass and a significant upregulation of AChRγ transcript and NCAM protein in the scAAV9(empty) treated mice compared with non-transgenic littermates. Notably, following the scAAV9_MCP1 injection, the TA muscle of C57 SOD1^{G93A} mice was less

atrophied compared with the scAAV9(empty) treated mice (25.9±2.3% of muscle mass lost compared with the non-transgenic mice) and did not upregulate NCAM suggesting the reduced denervation of the neuromuscular junction (NMJ) (Fig. 38A, C, D). Accordingly, the AChRγ transcript was significantly downregulated in treated mice compared with the control group (Fig. 38B).

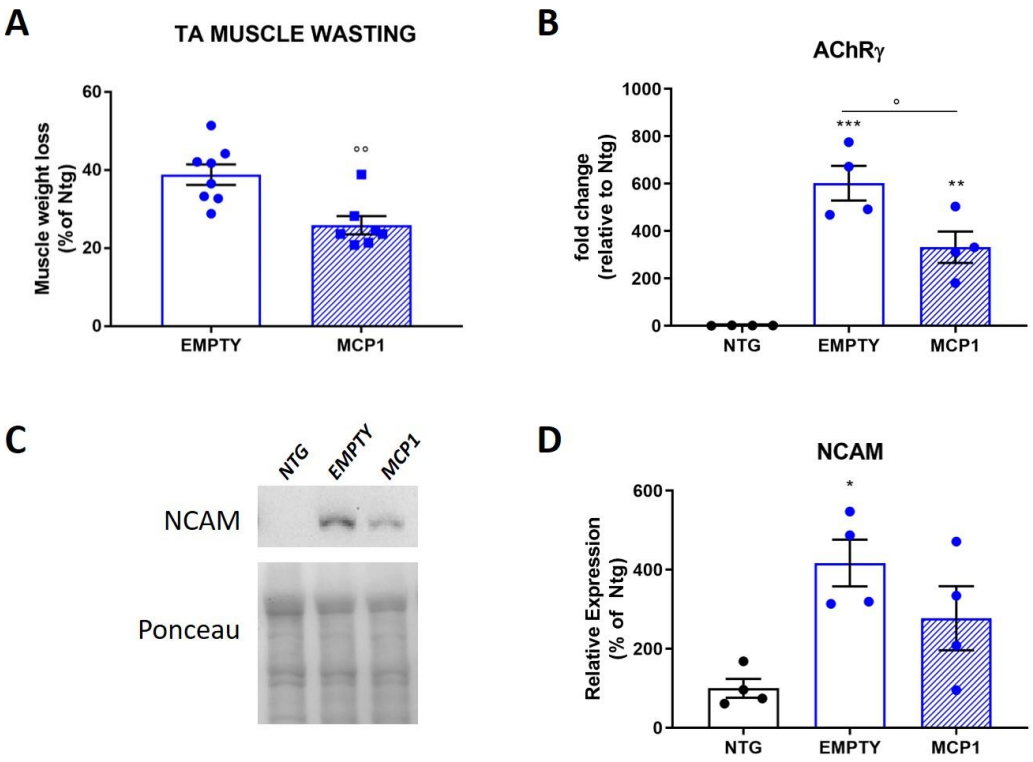

Figure 38: *(A) The analysis of the tibialis anterior (TA) muscle wasting revealed a significant reduction of the muscle atrophy in scAAV9_MCP1 treated mice compared with the control group. Data are expressed as mean±SEM (n=8 per experimental group). °°p<0.01 by unpaired t-test. (B) Real-time PCR analysis of the gamma subunit of the acetylcholine receptor (AChRγ) showed a significant upregulation of AChRγ transcript in scAAV9(empty) but not in scAAV9_MCP1 treated mice compared with the non-transgenic (Ntg) littermates. Data are normalised to βactin and expressed as mean±SEM (n=4 per experimental group). (C) Representative immunoblot analysis of the Neural Cell Adhesion Molecule (NCAM) in the TA muscle. (D) The densitometric analysis revealed a significant accumulation of NCAM in the TA muscle of scAAV9(empty) treated mice but not in the scAAV9_MCP1 treated mice compared with the non-transgenic (Ntg) littermates. Data and expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

Intriguingly, it has been reported that muscle wasting occurs with differential sensitivity between selective skeletal muscle fibre subtypes. Indeed, it has been reported that the fast-fatigable glycolytic muscle fibres are more susceptible to the atrophic phenomenon compared with the slow oxidative fibres (Wang and Pessin 2013). Notably, a metabolic dysregulation of skeletal muscle, characterised by the progressive loss of fast-twitch glycolytic fibres and a compensatory increase of slow-twitch oxidative fibres as the results of the mitochondrial deficit and oxidative stress, occurs in ALS patients and models (Telerman-Toppet and Coërs 1978; Palamiuc et al., 2015; Peggion et al.,

2017; Dobrowolny et al., 2018). Therefore, we analysed the expression of the succinate dehydrogenase (SDH) enzyme, a component of the mitochondrial respiratory chain complex II, as an index of the oxidative capacity of the TA muscle (Old and Johnson 1989; Martin et al., 1985). According to previous evidence (Palamiuc et al., 2015; Dobrowolny et al., 2018; Scaricamazza et al., 2020), the SDH staining showed a significant increase in the percentage of oxidative fibres in the ALS mice (Empty: 87.3±1.5%; MCP1: 74.8±1.9%) compared with the non-transgenic littermates (52.9±0.2%). Nevertheless, the scAAV9_MCP1 injection significantly reduced the SDH-positive oxidative fibres in the TA muscle of SOD1^{G93A} mice, thus preventing the metabolic degeneration occurring during ALS progression (Fig. 39A, B).

These beneficial effects seem to be strictly dependant from the chemokine induction, as demonstrated by the increased transcription level of MCP1 recorded in the TA muscle of treated mice compared with the control groups (Fig. 39C).

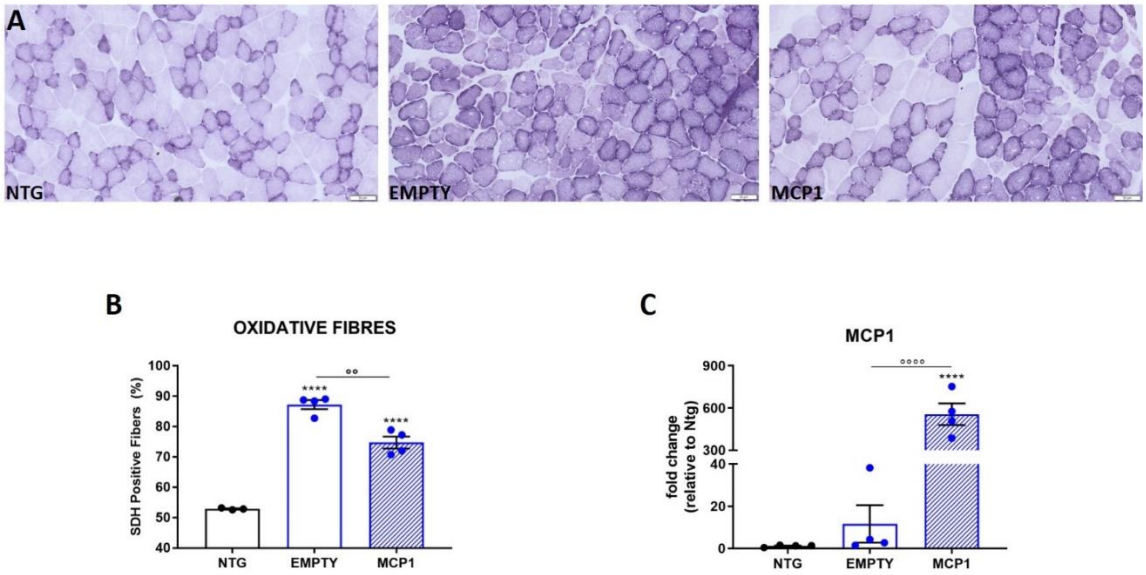

Figure 39: *(A) Confocal micrographs of coronal sections of the tibialis anterior (TA) muscle stained for the Succinic Dehydrogenase (SDH) enzyme to identify oxidative muscle fibres. (B) Imaging analysis revealed an increased percentage of oxidative fibres in the TA muscle of ALS mice, albeit significantly lower upon MCP1 induction. Data are expressed as mean±SEM (n=3 Ntg; n=4 SOD1^{G93A} mice). Scale bar= 50µm. (C) Real-time PCR analysis of MCP1 transcript revealed a significant upregulation of the chemokine in treated mice compared with the control groups. Data are normalised to βactin and expressed as mean±SEM (n=4 per experimental group). ****p<0.0001 Ntg Vs EMPTY or MCP1; °°p<0.01, °°°°p<0.0001 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

8.3.2 CHARACTERISATION OF THE RECRUITMENT OF THE IMMUNE CELLS AND THE INFLAMMATORY MILIEU IN THE TIBIALIS ANTERIOR MUSCLE

Several shreds of evidence have highlighted the pivotal role of MCP1 in driving the skeletal muscle regeneration thanks to its chemoattractant activity toward CCR2-expressing cells (Shireman et al., 2007; Contreras-Shannon et al., 2007; Lu et al., 2011b; Martinez et al., 2010; Oishi and Manabe 2018).

Interestingly, the kinetic of leucocytes accumulation within the injured skeletal muscle resembles the classical response to infection. The damage-associated molecular patterns (DAMPs) and cytokines released by necrotic cells attract neutrophils, which remove the fibre debris (Dumont et al., 2008) and foster the recruitment of macrophages and T lymphocytes (Yang and Hu 2018; Butterfield et al., 2006; Oishi and Manabe 2018; Rigamonti et al., 2013; Pizza 2008; Tidball 2017).

At 14weeks, the damaged muscle of $SOD1^{G93A}$ mice recruited neutrophils, as demonstrated by the massive expression of the proteolytic elastase enzyme (chymotrypsin-like serine-proteinase) (Okada 2017; Arecco et al., 2016) compared with the non-transgenic animals. Conversely, the induction of MCP1 early in the disease anticipated the physiological immune response within the skeletal muscles of ALS mice. Indeed, neutrophils were no longer present within the TA muscle of treated mice (Fig. 40B, D). In contrast, upon MCP1 induction, we recorded a remarked infiltration of phagocytic $CD68^{+}$ macrophage and T lymphocytes compared with the scAAV9(empty) treated mice (Fig. 40A, C).

Moreover, although the treatment did not significantly modify the extent of $CD8^{+}$ and $CD4^{+}$ T lymphocytes infiltration, the increased recruitment of the immunoregulatory T reg cells, which are pivotal in promoting skeletal muscle regeneration (Castiglioni et al., 2015; Schiaffino et al., 2017), was recorded compared with the scAAV9(empty) treated mice (Fig. 40E-G).

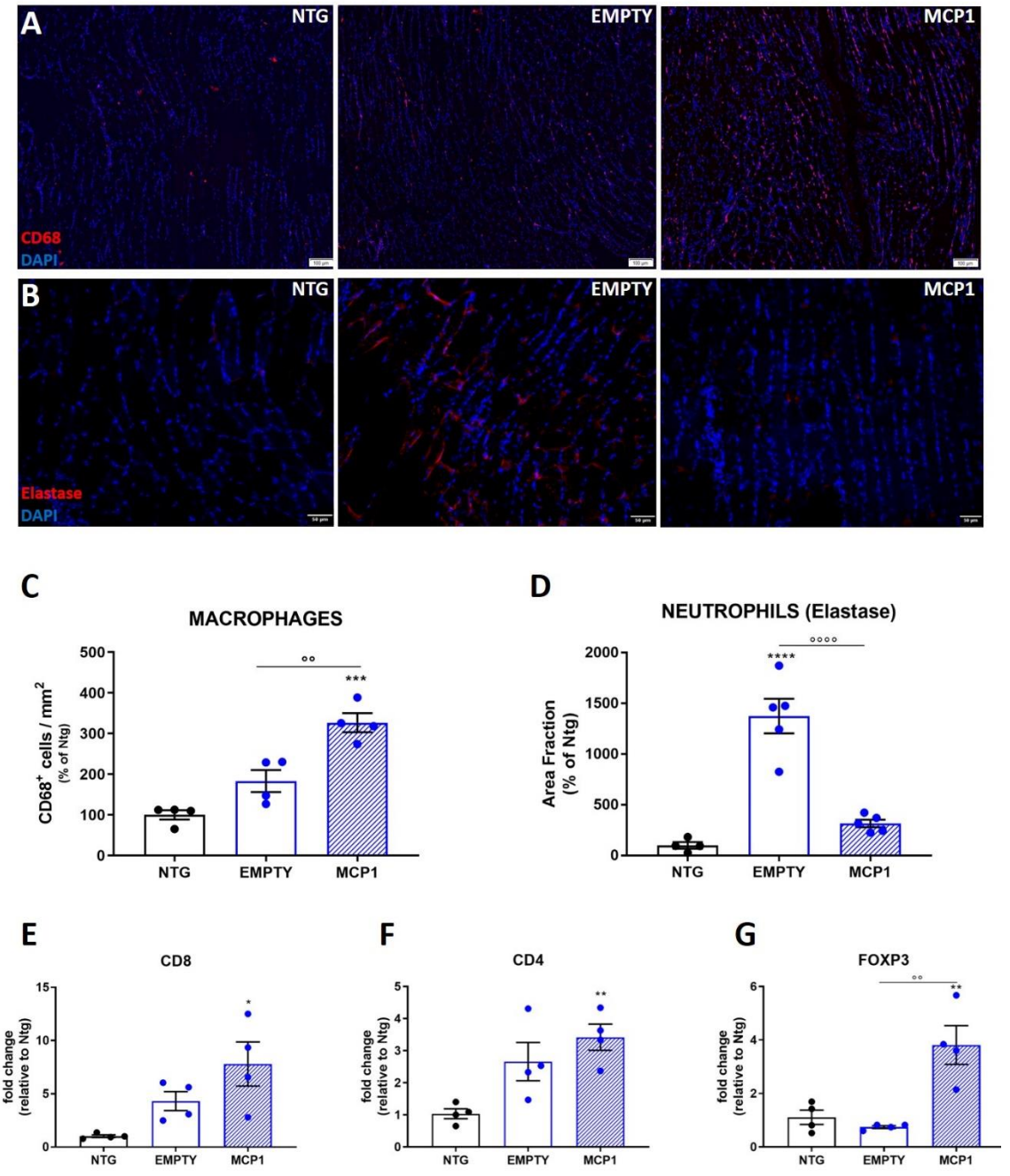

Figure 40: *(A, B) Confocal micrographs of longitudinal sections of tibialis anterior (TA) muscle stained with the CD68 phagocytic marker (A) or Elastase enzyme (B) and DAPI (nucleus). Imaging analysis revealed increased recruitment of macrophages (C) and a reduced presence of neutrophils (D) in the TA muscle of treated mice compared with the control group. Data are expressed as mean±SEM (n=4 per experimental group). A, scale bar= 100μm; B, Scale bar= 50μm. (E, G) Real-time PCR analysis of CD8α receptor, CD4α receptor and Foxp3 transcripts in the TA muscle of ALS mice and non-transgenic (Ntg) littermates. The scAAV9_MCP1 injection increased the infiltration of T lymphocytes in the TA muscle of ALS mice compared with the control groups. Data are normalised to βactin and expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Ntg Vs EMPTY or MCP1; °°p<0.01, °°°°p<0.0001 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

The ability of the infiltrated immune cells in governing the response of the skeletal muscle to injury is strictly dependent to their M1/M2 polarisation (Arnold et al., 2007; Kharraz et al., 2013; Tidball

2017; Tidball et al., 2014; Saclier et al., 2013b; Patsalos et al., 2017). Therefore, we analysed the inflammatory milieu of the TA muscle of SOD1^{G93A} mice at 14 weeks of age.

In line with the first phase of the immune response to damage (Yang and Hu 2018), we recorded a strong upregulation of the inflammatory marker TNFα in the TA muscle of scAAV9(empty) treated mice compared with the non-transgenic animals (Fig. 41A). Conversely, the leucocytes infiltrated in the hind paw muscle of scAAV9_MCP1 treated mice have already shifted toward the anti-inflammatory phenotype. Indeed, the transcription of TNFα was downregulated in favour of the increased expression of the M2 pro-regenerative marker Arginase 1 compared to the scAAV9(empty) treated mice (Fig. 41A, C, D).

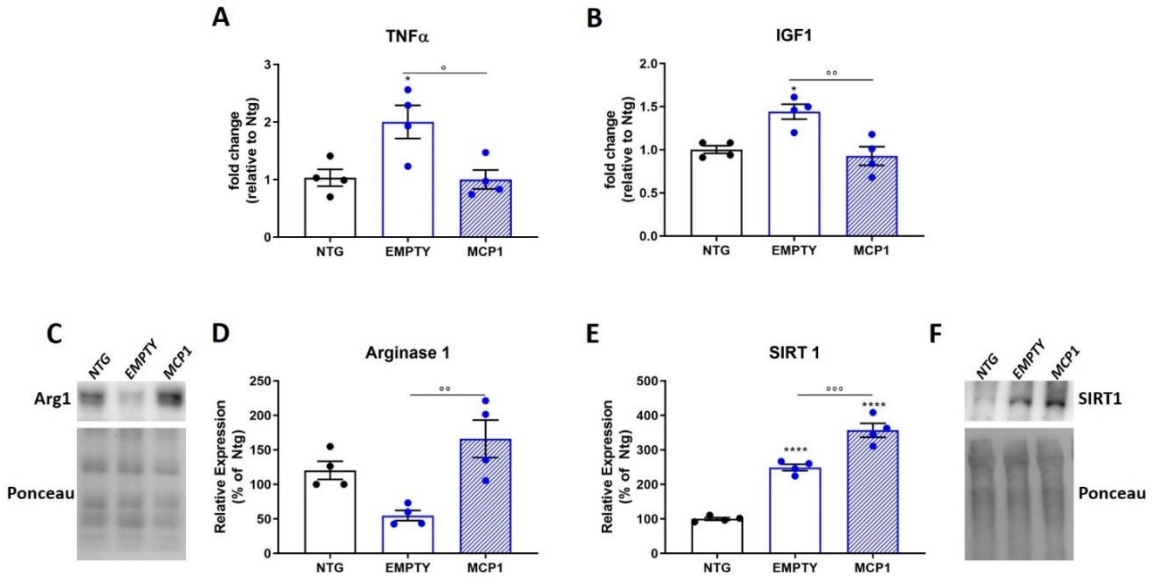

Figure 41: *(A, B) Real-time PCR analysis of tumour necrosis factor alpha (TNFα) and insulin-like growth factor 1 (IGF1) transcripts in the tibialis anterior (TA) muscle of ALS mice and non-transgenic (Ntg) littermates. The gene expression analysis showed significant downregulation of TNFα and IGF1 in the TA muscle of treated mice compared with the control group. Data are normalised to βactin and expressed as mean±SEM (n=4 per experimental group). (C-D) The immunoblot analysis revealed an increased expression of Arginase 1 (Arg1) and Sirtuin 1 (SIRT1) in the TA muscle of treated mice compared with the control group. Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05, °°p<0.01, °°°p<0.001 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

Coherently, IGF1 transcript resulted significantly downregulated in the hind paw muscle of treated mice compared with the control group (Fig. 41B), suggesting the complete switch of the infiltrated macrophages toward the anti-inflammatory phenotype (Tonkin et al., 2015). To verify this evidence, we analysed the expression of the NAD$^+$-dependent Sirtuin 1 (Sirt1) deacetylase, enzyme promoting both the myogenic activity of satellite cells (Cerletti et al., 2012; Rathbone et al., 2009) and the

macrophage shift toward the M2 phenotype by inhibiting NFκB signalling (Tonkin et al., 2012; Schug et al., 2010). Consistently, we found that Sirt1 was significantly expressed within the TA muscle of treated mice compared with controls (Fig. 41E, F).

8.3.3 CHARACTERISATION OF THE SATELLITE CELL-MEDIATED RESPONSE IN THE TIBIALIS ANTERIOR MUSCLE

Numerous evidence suggest the both the innate and the adaptive immune response actively influence the skeletal muscle regeneration directly governing the response of satellite cells in both acute and chronic injury (Deyhle and Hyldahl 2018; Madaro et al., 2019; Tidball and Villalta 2010). Therefore, we analysed the expression of two critical myogenic transcription factors: Pax7, the hallmark of satellite cells stemness (Mauro 1961), and Myogenin (MyoG), a marker of early commitment and differentiation (Cornelison and Wold 1997) in the TA muscle of 14 weeks-old mice.

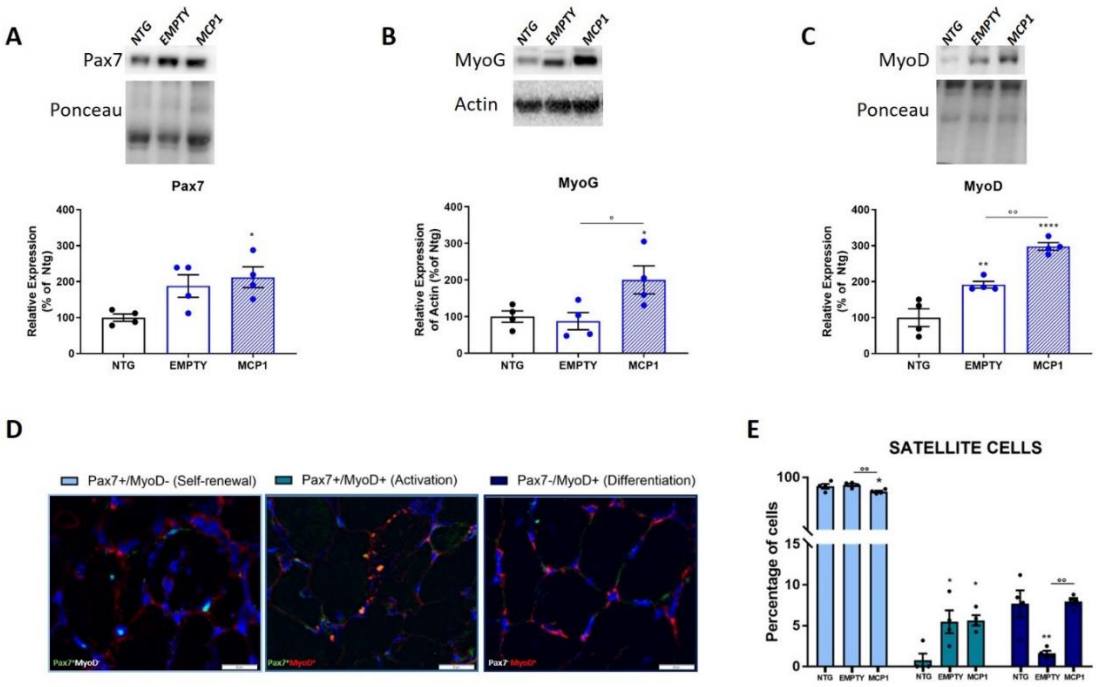

Figure 42: *(A-C) Immunoblot analysis of satellite cells transcriptional factors Pax7 (A), MyoG (B) and MyoD (C) in the tibialis anterior (TA) muscle of ALS mice and non-transgenic (Ntg) littermates. The densitometric analysis indicated an increased expression of these markers in treated mice compared with the control group. Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05, °°p<0.01 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis. (D) Confocal micrographs of coronal sections of TA muscle stained with Pax7 (red), MyoD (green) and DAPI (blue). Scale bar= 20µm. (E) Imaging analysis indicated a higher percentage of differentiating ($MyoD^+/Pax7^-$) satellite cells in the TA muscle of treated mice compared with the control group Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, Ntg Vs EMPTY or MCP1; °°p<0.01 EMPTY Vs MCP1 by Two-way ANOVA with Tukey's post-analysis.*

As shown in Figure 42, MyoG, but not Pax7, was significantly activated in the TA muscle of treated mice compared with the control group, suggesting that the early MCP1-mediated immune response promoted the myofiber shift towards the differentiation programme (Fig. 42A, B).

Moreover, the treatment strongly enhanced the activation of the myoblast determination protein 1 (MyoD) transcription factor in the TA muscle of ALS mice compared with the control groups (Fig. 42C), which is critical at defining the fate of the activated satellite cells. MyoD downregulation guarantees the preservation of the satellite cells pool, whereas its upregulation coincides to the ceasing in the Pax7 expression promoting the final commitment to myoblast (Relaix and Zammit 2012; Forcina et al., 2019). Accordingly, our data suggest an enhancement of the myogenic activity in the TA muscle of scAAV9_MCP1-treated mice since the early stage of the disease. In keeping with this, the histological evaluation of the satellite cells dynamic in the hindlimb muscle of $SOD1^{G93A}$ mice showed a reduction of quiescent ($Pax7^{+}/MyoD^{-}$) satellite cells and a significant increase in their differentiation ($Pax7^{-}/MyoD^{+}$) upon MCP1 induction (Fig. 42D, E).

Several shreds of evidence describe that, in resting muscles, the myonuclei are positioned at the periphery of the muscle fibres, to protect them from the force of contraction, and are distributed to maximise the distance to each other and to guarantee the transcription and translation machinery necessary to sustain the entire myofiber (Bruusgaard et al., 2003; Folker and Baylies 2013). However, during the regenerative process, muscle fibres undergo a series of architectural changes among which the positioning of the nucleus at their centre (Folker and Baylies 2013). According to the increased myogenic activity observed upon MCP1 induction, the histological analysis performed showed an increased density of muscle fibres characterised by the central location of the nucleus in the TA muscle of treated mice compared with the control groups (Fig. 43).

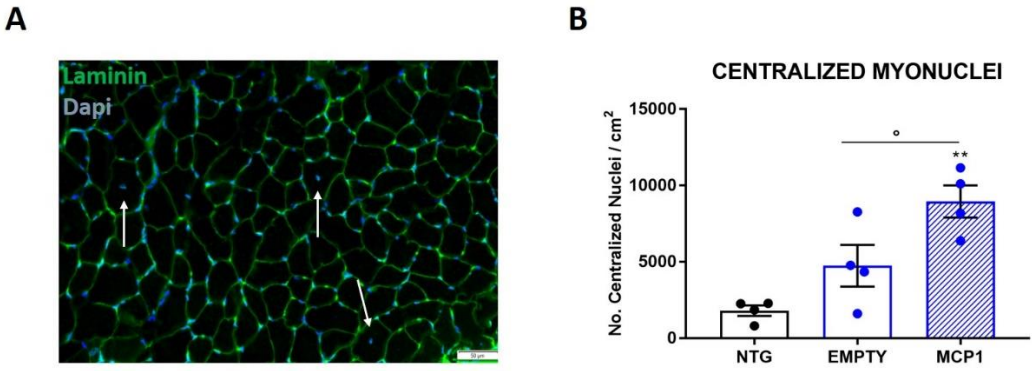

Figure 43: *(A) Confocal micrographs of coronal sections of the tibialis anterior (TA) muscle stained with Laminin (extracellular matrix) and DAPI (nucleus). Scale bar= 50µm. Regenerating muscle fibres display centrally located myonuclei (white arrow). (B) Imaging analysis indicated an increased density of muscle fibres characterised by the central location of the myonucleus in treated mice compared to the control groups. Data are expressed as mean±SEM (n=4 per experimental group). **p<0.01 Ntg Vs MCP1; °p<0.05 EMPTY Vs MCP1 by Two-way ANOVA with Tukey's post-analysis.*

8.4 ANALYSIS OF THE EARLY EFFECT OF MCP1 INDUCTION IN THE SCIATIC NERVE

8.4.1 VALIDATION OF MCP1 INDUCTION, IMMUNE CELLS INFILTRATION AND SCHWANN CELL-MEDIATED RESPONSE

At the disease onset, the C57 SOD1^{G93A} mice strongly activated the MCP1-mediated pathway to recruit macrophages and T lymphocytes within degenerating motor axons (Nardo et al., 2016b). However, here, we did not record any difference in the transcription of the CD68 phagocytic marker between the two groups of ALS mice, although MCP1 was significantly upregulated six weeks after the scAAV9_MCP1 injection (Fig. 44A, B). Besides, the gene expression analysis of the CD8 cytotoxic T cell marker and the pro-inflammatory cytokine TNFα showed that the sciatic nerve of ALS mice did not exhibit any sign of inflammation compared with the non-transgenic littermates at the presymptomatic stage of the disease even upon MCP1 induction (Fig. 44C, D).

These data suggest that the extent of impairment of the sciatic nerves of SOD1^{G93A} mice at 14 weeks (i.e. ~2 weeks before the onset of motor symptoms) is not sufficient to promote the recruitment of haematogenous immune cells, which instead showed a considerable influence late in the disease (Nardo et al., 2016b).

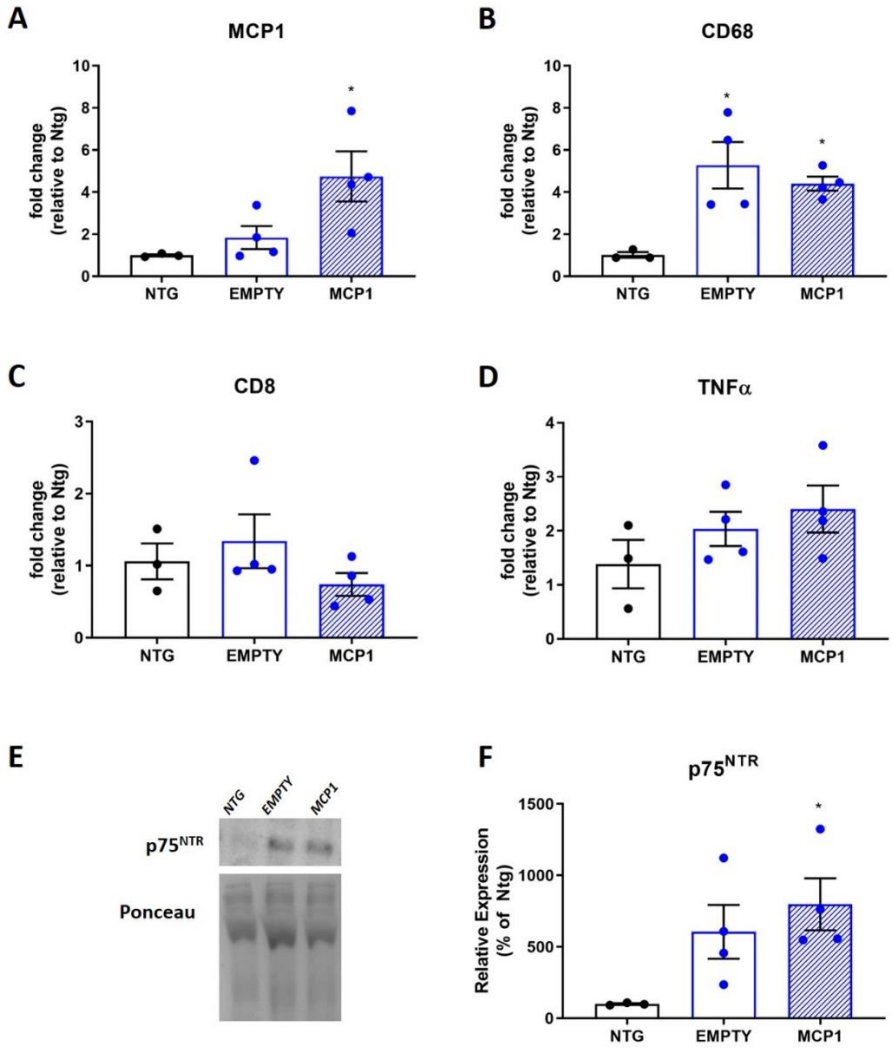

Figure 44: *(A, D) Real-time PCR analysis of MCP1 (A), the CD68 phagocytic marker (B), CD8α receptor (C) and tumour necrosis factor alpha (TNFα) (D) transcripts in the sciatic nerve of ALS mice and non-transgenic (Ntg) littermates. Although MCP1 mRNA was significantly upregulated in treated mice compared with the control groups (A), no difference in the transcription of CD68 was recorded between the two groups of ALS mice (B). Moreover, no variation in the transcriptional levels of CD8α receptor (C) and TNFα (D) was recorded in the three experimental groups. Data are normalised to βactin and expressed as mean±SEM (n=4 per experimental group). (E, F) The immunoblot analysis revealed a higher expression of p75^{NTR} in the sciatic nerve of treated mice compared with the control groups. Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05 Ntg Vs EMPTY or MCP1 by ANOVA with Tukey's post-analysis.*

The Schwann cells (SCs) are the first line response following a peripheral injury that precede and promote the infiltration of haematogenous immune cells through the release of chemoattractant factors, including MCP1 (Gaudet et al., 2011; Jessen and Mirsky 2019).

We found an increased expression of p75^{NTR} in the sciatic nerves of scAAV9_MCP1 treated mice compared to the control groups (Fig. 44E, F), indicating an early de-differentiation of SCs toward an immature non-myelinating phenotype, which is prodromal to their proliferation/regeneration (Jessen and Mirsky 2008). This evidence suggests that in the scAAV9_MCP1 treated mice the SCs are better equipped to respond to injury compared with the control group (Deng et al., 2018).

8.5 ANALYSIS OF THE EARLY EFFECT OF MCP1 INDUCTION IN THE LUMBAR SPINAL CORD

8.5.1 ANALYSIS OF THE MOTOR NEURON SURVIVAL AND GLIA CELLS ACTIVATION IN THE LUMBAR SPINAL CORD

We previously showed that the intramuscular injection of scAAV9 specifically transduces the MN perikaryon (Chapter V). Therefore, we first examine the MCP1 transcript in the lumbar spinal cord.

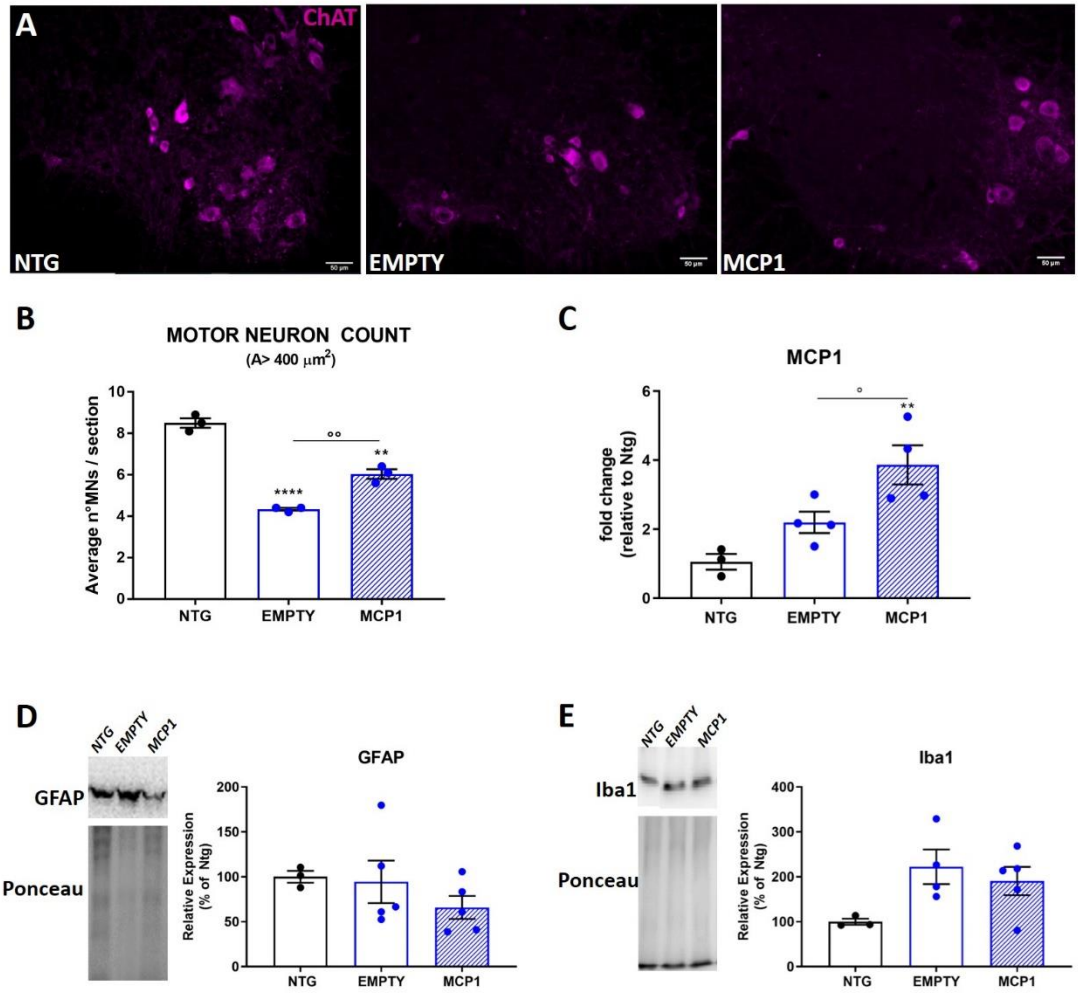

Figure 45: *(A) Confocal micrographs of coronal sections of the lumbar spinal cord of ALS mice and non-transgenic (Ntg) littermates stained with Choline Acetyltransferase (ChAT). Scale bar= 50µm. Data are expressed as mean±SEM (n=3 per group). (B) The scAAV9_MCP1 injection reduced the motor neuron loss treated mice compared with the control group. (C) The real-time PCR analysis of MCP1 transcript in the lumbar spinal cord showed a significant upregulation of the chemokine in the lumbar spinal cord of treated mice compared with the control group. Data are normalised to βactin and expressed as mean±SEM (n=4 per group). (D, E) The immunoblot analysis of the glial fibrillary acidic protein (GFAP) and ionised calcium-binding adapter molecule 1 (Iba1) in lumbar spinal cord extracts did not show any significant difference in the three experimental groups. Data are expressed as mean±SEM. **p<0.01, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05, °°p<0.01 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

The gene expression analysis showed a greater upregulation of the chemokine in the CNS of treated mice compared with the control group (Fig. 45C). Moreover, our findings revealed that the MCP1

induction exerted a beneficial effect in the lumbar spinal cord, where MNs resulted significantly protected compared to the control group (Fig. 54A, B). This result is in line with the pleiotropic neuroprotective role of the chemokine observed in the neurologic context (Papa et al., 2018; Locatelli et al., 2012).

We previously found that glial cells were not significantly activated in C57 $SOD1^{G93A}$ mice compared with non-transgenic littermates at the presymptomatic stage of the disease (Marino et al., 2015). Here, we confirmed this evidence observing that the induction of a pro-inflammatory chemokine within MN did not increase the gliosis within the lumbar spinal cord of ALS mice, as demonstrated by the unvaried expression of GFAP and Iba1 among the selected experimental groups at 14 weeks (Fig. 45D, E).

8.5.2 ANALYSIS OF THE INFLAMMATORY MILIEU IN THE LUMBAR SPINAL CORD

The data collected at the symptomatic stage of the disease showed that the specific MCP1 induction within MNs was able to modulate the neuroinflammatory phenomenon in ALS mice (section 7.5). Therefore, we investigated whether the immunomodulatory activity of the chemokine was already detectable a few weeks upon the scAAV9_MCP1 injection.

We found that the MCP1 induction within MNs was able to shift the glial fingerprint towards the M2 anti-inflammatory phenotype, as demonstrated by the upregulation of Arginase 1 and IL4 compared with the scAAV9(empty) treated mice (Fig. 46A, B). Moreover, our analysis revealed an attenuation in the expression of the pro-oxidative and inflammatory marker $gp91^{PHOX}$ upon MCP1 induction (Fig. 456). In line with the moderate activation of glial cells during this stage (Marino et al., 2015), no difference was observed in the transcription level of the IL1β chemokine between the three experimental groups (Fig. 46D).

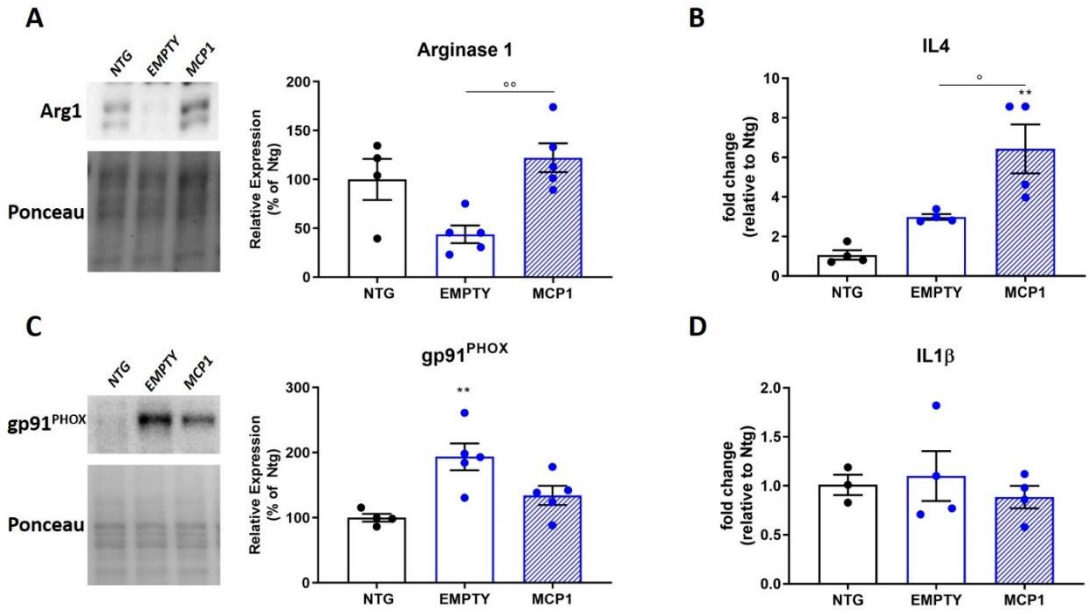

Figure 46: *(A, C) The immunoblot analysis showed a significant upregulation of Arginase 1 (A) and reduced expression of the NADPH oxidase subunit (gp91^{PHOX}) (C) in the lumbar spinal cord of treated mice compared with the control group. Data are expressed as mean±SEM (n=4 Ntg; n=5 SOD1^{G93A} EMPTY or MCP1). (B, D) Real-time analysis of interleukin 4 (IL4) and interleukin 1 beta (IL1ϐ) transcripts in the lumbar spinal cord of ALS mice and non-transgenic (Ntg) littermates. The gene expression analysis showed a significant upregulation of IL4 (B), but not IL1ϐ (C) in treated mice compared with the control group. Data are normalised to ϐactin and expressed as mean±SEM (n=4 per group). **p<0.01, Ntg Vs EMPTY or MCP1; °p<0.05, °°p<0.01 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

8.6 DISCUSSION

ALS is considered a non-cell autonomous disease, in which other (resident or infiltrating) cell types besides MNs actively participate to the disease progression (Ilieva et al., 2009; Boillée et al., 2006a). Indeed, immune cells infiltration has been reported in both central and peripheral compartment of ALS patients and models (Chiu et al., 2009; Schreiber et al., 2019; Engelhardt et al., 1993; Henkel et al., 2004; Appel et al., 2010). Notably, axonal degeneration, destruction of nerve terminals and muscle atrophy are early events in the disease pathogenic cascade, anticipating the MN degeneration and the onset of motor symptoms (Clark et al., 2016; Azzouz et al., 1997; Fischer et al., 2004). This evidence has led to ALS being recognised as a distal axonopathy, whereby skeletal muscle contributes to a retrograde signalling cascade that affects MNs (Moloney et al., 2014; Dadon-Nachum et al., 2011).

The role of inflammatory mechanisms in influencing the degeneration/regeneration of the peripheral compartment is still partially known in comparison with the understanding of inflammation involving glial and immune cells in the CNS. However, studies in rodent models of

the disease recently reported that successful axon and muscle regeneration depends on the coordinated efforts of immune cells which, besides removing the cellular debris, led the establishment of a permissive milieu to wound healing (Deng et al., 2018; Van Dyke et al., 2016; Nardo et al., 2016b). This evidence suggested a protective role of the immune response in the peripheral compartment of ALS.

The intramuscular injection of the scAAV9_MCP1 prevents the degeneration of the skeletal muscles of SOD1^{G93A} mice at the early stage of the disease anticipating the peripheral immune response

The analysis performed at the 14 weeks (i.e. ~2 weeks before the overt muscle strength impairment) showed that C57 SOD1^{G93A} mice, in concomitance with the loss of ~40% of the TA muscle mass, launched the inflammatory immune response activating the resident macrophages and recruiting neutrophils from circulation. As indicated by the fine kinetic governing the infiltration of the immune cells within the injured muscle (Yang and Hu 2018; Tidball 2017; Oishi and Manabe 2018), the chemotactic gradient (MCP1, CXCL2, GM-CSF, etc..) established within the injured muscle recruits neutrophils (Peterson and Pizza 2009; Shireman et al., 2007), which are the first non-resident cells entering within the damage site to amplify the inflammation and promote the recruitment of haematogenous macrophages (Teixeira et al., 2003; Tidball 1995). This evidence indicated that, in comparison to the extent of damage occurred in the TA muscle at 14 weeks, the pro-regenerative immune response of ALS mice is delayed and therefore inadequate to counteract the progressive denervation atrophy of the hind limb muscles. Conversely, the early induction of MCP1 anticipated the recruitment of the immune cells and this translated in remarkable preservation of the skeletal muscles of ALS mice, as demonstrated by the reduced denervation atrophy and metabolic dysregulation recorded in the TA muscle of treated mice compared with the control group. Indeed, the data collected showed that a “second wave” of immune cell infiltration, in which neutrophils give way to macrophages and T lymphocytes (Yang and Hu 2018; Tidball 2017; Oishi and Manabe 2018), characterised the TA muscle of treated mice six weeks after the scAAV9_MCP1 injection. Notably, our analysis revealed an increased infiltration of T reg cells, which

repress the inflammation sustaining the switch of M1 phagocytic macrophages towards the M2 myogenic pro-regenerative phenotype (Schiaffino et al., 2017; Villalta et al., 2014). Accordingly, TNFα and IGF1 were downregulated whereas Sirt1 was increased upon MCP1 induction, indicating that the phenotypic switch of macrophages has already occurred in the TA muscle of 14 weeks old treated mice (Tonkin et al., 2015; 2012).

Noteworthy, Sirt1 also sustains the myogenic activity (Rathbone et al., 2009; Tonkin et al., 2012), which is increased in the TA muscle of treated mice by virtue of the inductive action of the T reg cells and M2 macrophages on myogenic progenitor cells (Castiglioni et al., 2015; Tidball et al., 2014; Tidball 2017; Arnold et al., 2007), thus translating in the muscle regeneration.

Therefore, the data collected showed that the early induction of MCP1 anticipated the immune-mediated regeneration of the skeletal muscle of ALS mice, resulting in their preservation from the denervation atrophy.

The intramuscular injection of the scAAV9_MCP1 in SOD1^{G93A} mice did not modify the nerve response to damage at the presymptomatic stage of the disease

The analysis of the sciatic nerve of ALS mice did not show any notable difference following the MCP1 induction. Although the treatment increased the expression of p75^{NTR}, suggesting a better ability of treated mice to respond to the nerve damage (Deng et al., 2018), it did not modify the extent of the immune response. Indeed, differently from the scenario visible at the disease onset (Nardo et al., 2016b), we did not observe cytotoxic T cells infiltration either any sign of inflammation in both groups of ALS mice. These data suggest that at 14 weeks (i.e. ~2 weeks before the appearance of the motor dysfunction) the peripheral nerves of ALS mice are not damaged enough to require either the activation of the immune-mediated response or its enhancement through the MCP1 induction.

Our data are in line with the dying-back degeneration theory of the neuromuscular system in ALS (Dadon-Nachum et al., 2011; Clark et al., 2016) surmising that the skeletal muscle is the first compartment affected by the disease (Loeffler et al., 2016; Clark et al., 2016). Indeed, we showed that, during the early stages of the disease, a significant and beneficial immune response occurred

exclusively in the hind limb muscles of SOD1^{G93A} mice. Accordingly, it has been suggested that the peripheral nerve inflammation does not initiate the degenerative phenomenon in ALS but represents a response to the skeletal muscle degeneration (Kano et al., 2012).

The intramuscular injection of the scAAV9_MCP1 exerts a protective effect in the CNS of SOD1^{G93A} mice at the presymptomatic stage of the disease

Several pieces of evidence depicted MCP1 as a neurotoxic factor in ALS, inasmuch produced by activated microglia (Sargsyan et al., 2009; Henkel et al., 2006). However, we previously reported an increased expression of the chemokine in the MNs perikaryon of slow progressing compared with fast progressing SOD1^{G93A} mice, suggesting an intrinsic protective role of MCP1 in the CNS of ALS mice (Nardo et al., 2013).

Corroborative evidence demonstrated that MCP1 exerts a neuroprotective effect in rodent models of spinal cord injury directly acting on MN and also by promoting the polarisation of the recruited macrophages toward the M2-phenotype (Papa et al., 2018; Matsubara et al., 2015; Kwon et al., 2015; Niemi et al., 2016). Although haematogenous monocytes do not infiltrate the CNS of SOD1^{G93A} mice (Chiu et al., 2009; Kunis et al., 2015; Chiot et al., 2020), we showed that boosting MCP1 specifically within MNs promoted the M2 fingerprint in resident microglia, which resulted in the reduction of the neuroinflammatory phenomenon and thus in MNs preservation.

In conclusion, the data collected at the presymptomatic stage of the disease showed that the early induction of MCP1 alongside the motor unit of ALS mice could exert a dual protective effect. In the periphery, through its “classic” chemotactic activity toward leucocytes, MCP1 induction anticipated the physiologic immune response in the skeletal muscle of ALS mice, thus promoting the tissue regeneration and the preservation of the muscle strength. In the CNS, the MCP1 induction counteracted the neuroinflammatory phenomenon and protected MNs from degeneration through an immune-unrelated pleiotropic activity.

RESULTS

Chapter IX

Evaluation of the effect of scAAV9_MCP1 i.m. injection in slow progressing ALS mice: the involvement of forelimb motor units

9.1 BACKGROUND and AIM

Ever since its development, mSOD1 transgenic mouse has been the most widely used animal model of ALS. Indeed, it faithfully recapitulates many of the pathological features of the disease (Philips and Rothstein 2015).

However, differently from ALS patients, mSOD1 mice first develop hindlimb tremors, then progressive hindlimb weakness with rapidly deteriorating gait, eventually culminating in the paralysis of one or both hindlimbs (Gurney 1997; Gurney et al. 1994; Bruijn et al., 1997; Bendotti and Carrì 2004). Forelimbs function remains comparatively spared throughout the disease progression, indicating a distinct susceptibility of the upper motor unit in mSOD1 mice (Bruijn et al., 1997; Nardo et al., 2018; Schäfer and Hermans 2011). Accordingly, considerable evidence highlighted fundamental differences in the hindlimbs compared with forelimbs motor units response to the disease (Beers et al., 2011b; Capitanio et al., 2012; Clark et al., 2016). However, the comprehension of the mechanisms underlying the ascending paralysis characterising the mSOD1 mice is still unclear.

These observations highlighted the importance of the forelimbs contribution in the disease progression of ALS mice, particularly in the advanced stage of the disease. Accordingly, we have recently demonstrated that the preservation of the upper motor units functions actively influenced the disease duration and the overall survival of $SOD1^{G93A}$ mice (Nardo et al., 2018).

The behavioural analysis herein performed showed that the scAAV9_MCP1 injection in both hindlimbs and forelimbs muscles ameliorated the motor ability of C57 $SOD1^{G93A}$ mice until the symptomatic stage, suggesting a protective effect also within the upper motor units. Moreover, in light of tardive involvement of the forepaws in the disease course, the analysis of the upper motor units might be helpful to shed light on the precocious alterations and the pro-regenerative response activated by $SOD1^{G93A}$ mice within the neuromuscular compartment. Accordingly, the next section of this Thesis aimed to characterise the effect of the MCP1 induction alongside the upper motor unit of C57 $SOD1^{G93A}$ mice.

9.2 EXPERIMENTAL DESIGN

An extensive histological and biochemical/molecular analysis was performed at the Triceps Brachii (TB) muscle and cervical spinal cord level of scAAV9_MCP1 treated mice and relative controls (empty vector) at both the presymptomatic (14 weeks) and the clear symptomatic (20 weeks) stage of the disease.

9.3 ANALYSIS OF THE EFFECT OF MCP1 INDUCTION IN THE TRICEPS BRACHII MUSCLE

9.3.1 ANALYSIS OF THE DENERVATION ATROPHY OF THE TRICEPS BRACHII MUSCLE AT THE PRESYMPTOMATIC AND SYMPTOMATIC STAGE OF THE DISEASE

The clinic impairment of the forepaws appears late in the disease of SOD1^{G93A} mice (Schäfer and Hermans 2011). However, likewise the hindlimbs, alterations in the forepaws could be detectable before the observation of evident motor impairment (Clark et al., 2016). Therefore, we first characterised the degeneration of the TB muscle in both treated and control C57 SOD1^{G93A} mice at the presymptomatic and symptomatic stage of the disease.

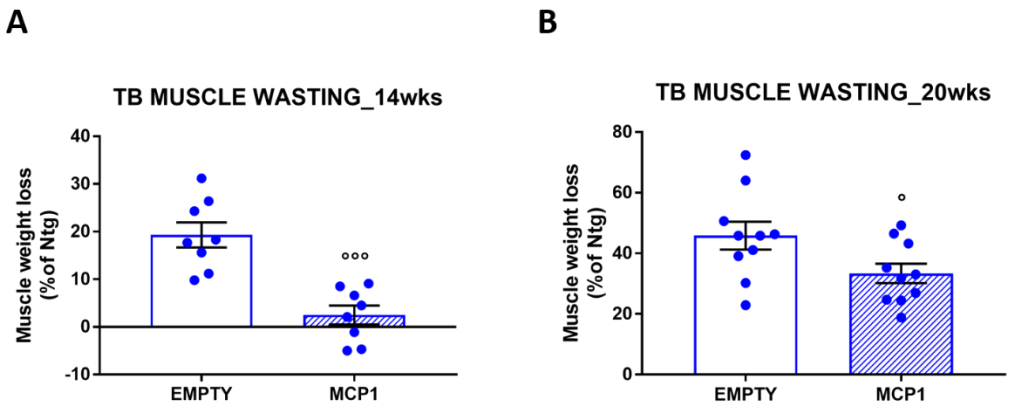

Figure 47: *(A, B) The analysis of the triceps brachii (TB) muscle wasting revealed a significant reduction of the muscle atrophy in scAAV9_MCP1 treated mice compared with the control group at both presymptomatic (14wks) and symptomatic (20wks) stage of the disease. Data are expressed as mean±SEM (14wks: n=8 per experimental group; 20wks: n=10 per experimental group). °p<0.05, °°°p<0.001 by unpaired t-test.*

The recording of the muscle weight showed that at 14 weeks the TB muscle of ALS mice lost the 19.3±2.6% of its mass compared with the non-transgenic littermates (Fig. 47A), which increased at the 46.8±4.1% at 20 weeks (Fig. 47B). Notably, at both time points, the induction of MCP1 significantly preserved the TB muscle of ALS mice from the atrophic phenomenon reducing the

muscle mass loss compared with the non-transgenic littermates at the 2.5±1.9% and 31.3±2.6% respectively (Fig. 47).

Suitably, our analysis showed that, at 14 weeks, the AChRγ transcript is significantly upregulated in the TB muscle of ALS mice, indicating the alteration of the NMJs before the appearance of any clinical sign of motor impairment. However, in the treated mice, we recorded a slightly increased of the AChRγ transcript, suggesting an MCP1-mediated protective effect on the forelimb muscles even starting from the presymptomatic stage of the disease (Fig. 48A). Nevertheless, we did not record any significant difference in the expression of the Neural Cell Adhesion Molecule (NCAM) in the two experimental groups of SOD1^{G93A} mice (Fig. 48C). Intriguingly, the beneficial effect of the MPC1 induction was more evident in the late stage of the disease, where we recorded a significant downregulation of AChRγ transcript and NCAM expression compared with the scAAV9(empty) treated mice (Fig. 48B, D).

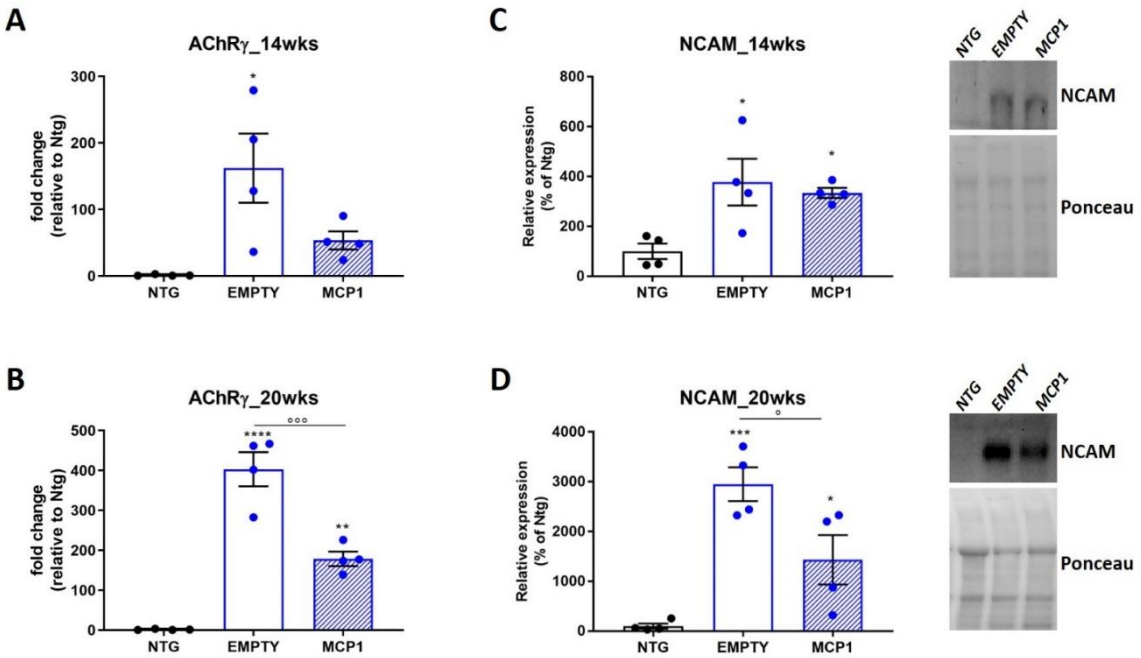

Figure 48: *(A, B) Real-time PCR analysis of acetylcholine receptor gamma-subunit (AChRγ) transcript in the triceps brachii (TB) muscle at the presymptomatic (A) and symptomatic (B) stage of the disease. Data are normalised to βactin and expressed as mean±SEM (n=4 per experimental group). (C, D) Immunoblot analysis of the Neural Cell Adhesion Molecule (NCAM) in the TB muscle at 14 weeks (C) and 20 weeks (D). Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05, °°°p<0.001 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

9.3.2 ANALYSIS OF THE RECRUITMENT OF THE IMMUNE CELLS AND INFLAMMATORY MILIEU AT THE PRESYMPTOMATIC AND SYMPTOMATIC STAGE OF THE DISEASE

We next analysed the extent of MCP1 induction in the TB muscle six weeks and twelve weeks after the scAAV9_MCP1 i.m. injection. The gene expression analysis showed that MCP1 resulted significantly upregulated in the treated mice compared with the control group with the same extent at both the considered time points (Fig. 49A, B).

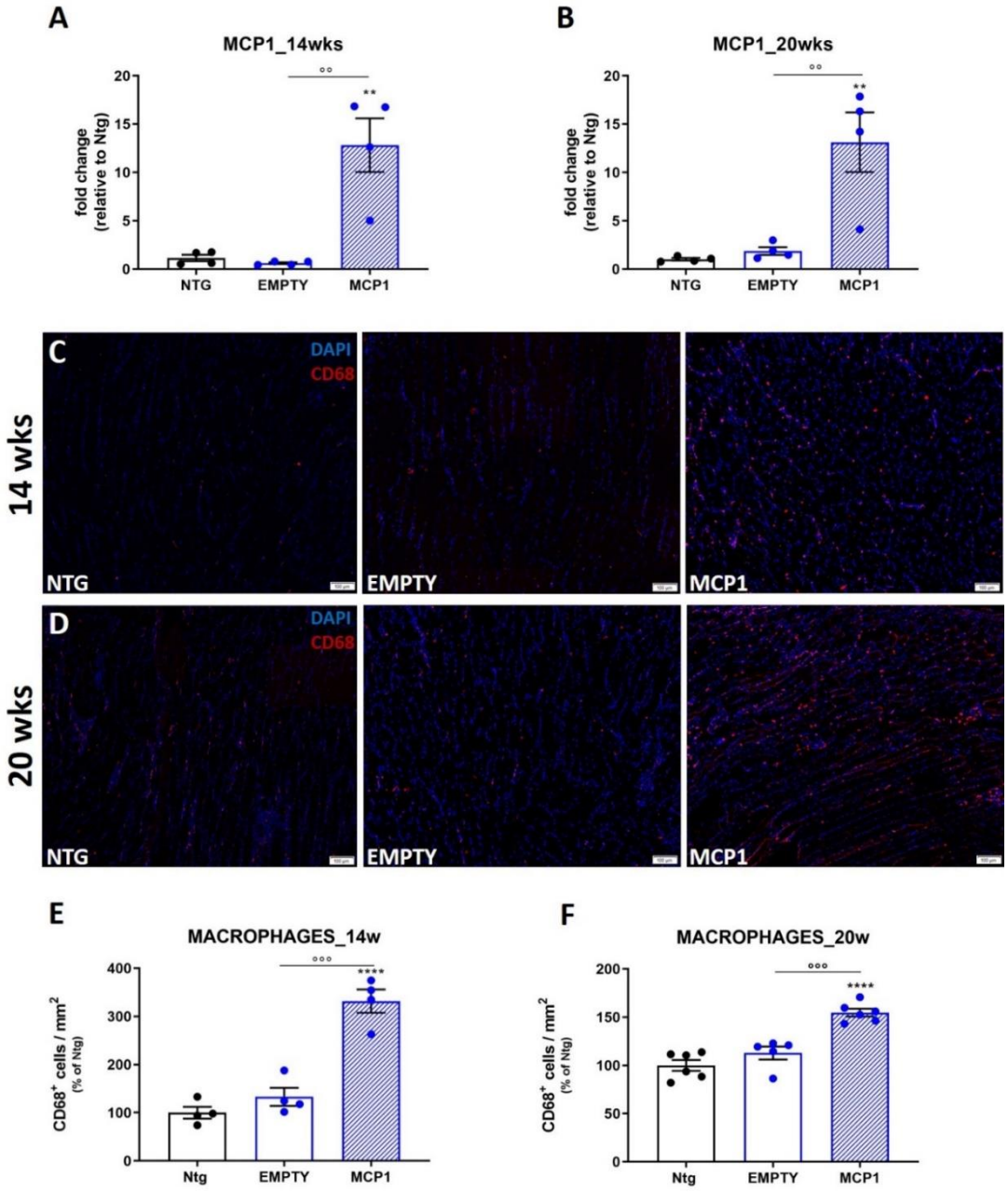

Figure 49: *(A, B) Real-time PCR analysis of MCP1 transcript in the triceps brachii (TB) muscle at the presymptomatic (A) and symptomatic (B) disease stage. Data are normalised to βactin and expressed as mean±SEM (n=4 per group). (C, D) Confocal micrographs of longitudinal sections of the TB muscle stained with the CD68 phagocytic marker and DAPI*

*(nucleus). Scale bar= 100µm. (E, F) Imaging analysis revealed increased recruitment of macrophages in the TB muscle of treated mice compared with the control group at both time points. Data are expressed as mean±SEM (14wks: n=4 per group; 20wks: n=6 per group). ****p<0.0001 Ntg Vs MCP1; °°°p<0.001 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

Suitably, macrophages recruitment was significantly increased in the TB muscle of treated mice compared with the control group at both time points (Fig. 49C-F). Nevertheless, at 14 weeks, the MCP1 induction fostered the infiltration of cytotoxic $CD8^+$ T cells (but not $CD4^+$ cells and $FoxP3^+$ T reg cells) (Fig. 50A-C); whereas, at 20 weeks, the scAAV9_MCP1 treated mice showed a trend in the reduction of lymphocytes recruitment (Fig. 50D-F). On the contrary, scAAV9(empty) treated mice did not recruit lymphocytes at the presymptomatic stage of the disease (Fig. 50A-C). In contrast, a significant infiltration of T cells and immunomodulatory lymphocytes was recorded in the mSOD1 mice at the symptomatic stage, arguably to counteract the denervation atrophy occurred and promote the skeletal muscle regeneration (Fig. 50D-F).

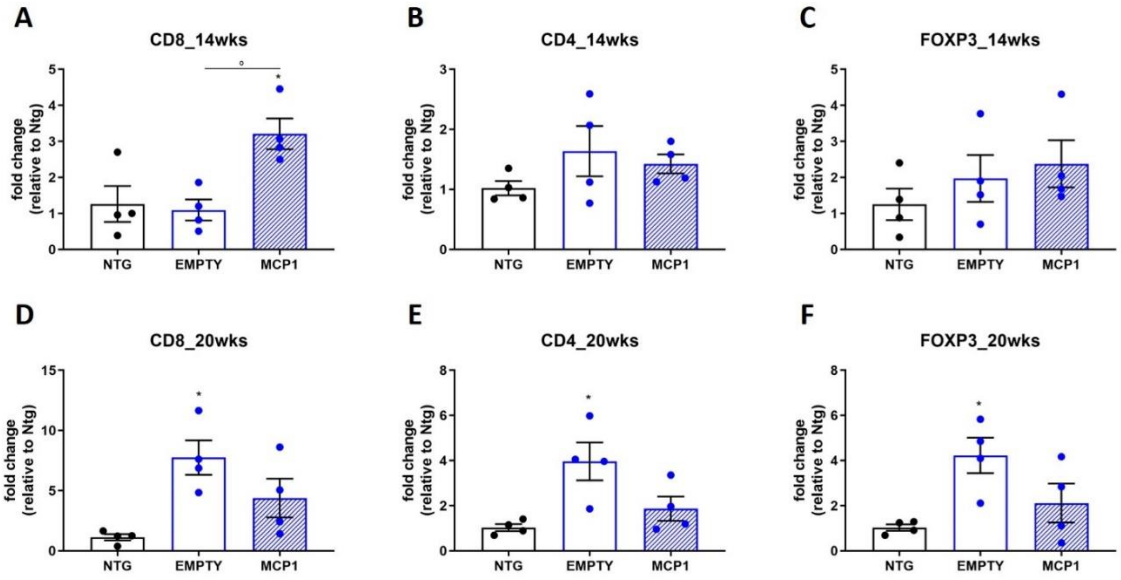

Figure 50: *Real-time PCR analysis of CD8α receptor, CD4α receptor and Foxp3 transcripts in the triceps brachii (TB) muscle of ALS mice and non-transgenic (Ntg) littermates at the presymptomatic (A-C) and symptomatic (D-F) stage of the disease. Data are normalised to βactin and expressed as mean±SEM (n=4 per experimental group). *p<0.05 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

Based on this evidence, we next analysed the M1/M2 polarisation of the immune cells recruited within the forepaw muscle of ALS mice following MCP1 induction. At 14 weeks, TNFα was significantly upregulated in the TB muscle of treated mice compared with controls while no difference in the expression of Arginase 1 was found between the two groups of ALS mice (Fig. 51A, E), suggesting a massive infiltration of M1 polarised leukocytes six weeks after the scAAV9_MCP1

injection. Notably, our analysis revealed a significant increase in the transcription level of IGF1 (Fig. 51C), indicating the switching of the infiltrated M1 cells toward the anti-inflammatory phenotype (Tonkin et al., 2015). Accordingly, at the symptomatic stage of the disease, TNFα was downregulated whereas Arginase 1 was increased in the treated mice compared with the control group (Fig. 51B, F), suggesting the establishment of an anti-inflammatory milieu twelve weeks after the scAAV9_MCP1 injection. Suitably, we did not record any difference in the transcription of IGF1 among the experimental groups at the symptomatic disease stage (Fig. 51D).

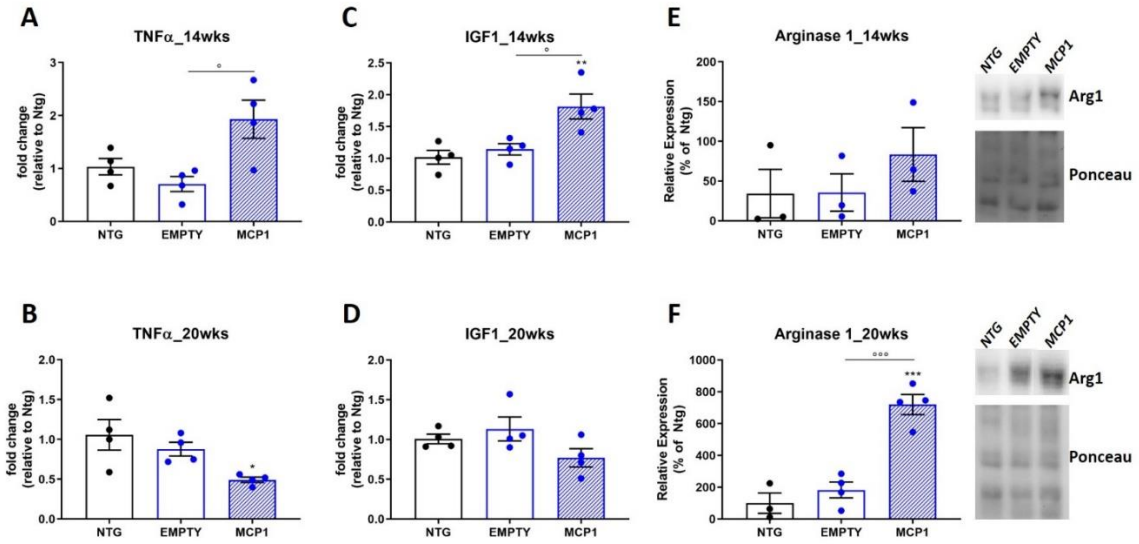

Figure 51: *(A-D) Real-time PCR analysis of tumour necrosis factor alpha (TNFα) and insulin-like growth factor 1 (IGF1) transcripts in the triceps brachii (TB) muscle of ALS mice and non-transgenic (Ntg) littermates at the presymptomatic (14 weeks) and symptomatic (20 weeks) stage of the disease. The gene expression analysis revealed a significant upregulation of TNFα and IGF1 transcripts in the treated mice compared with the control group at the presymptomatic stage of the disease (A, C). Conversely, an opposite trend was observed at the symptomatic stage of the disease (B, D). Data are normalised to βactin and expressed as mean±SEM (n=4 per experimental group). (E, F) Immunoblot analysis performed in the TB muscle extract showed a significant expression of Arginase 1 (Arg1) in treated mice compared with the control group at the symptomatic (F) but not at the presymptomatic (E) stage of the disease. Data are expressed as mean±SEM (n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001 Ntg Vs MCP1; °p<0.05, °°°p<0.001 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

9.3.3 CHARACTERISATION OF THE SATELLITE CELL-MEDIATED RESPONSE AT THE PRESYMPTOMATIC AND SYMPTOMATIC STAGE OF THE DISEASE

It has been reported that the inflammatory polarisation of the infiltrating immune cells actively influences the fate of the myogenic progenitor cells (MPCs, a.k.a. satellite cells) (Tidball 2017; Yang and Hu 2018). Therefore, we analysed the effect of the MCP1-mediated immune cells infiltration on the activity of the satellite cells in the TB muscle of ALS mice at both 14 weeks and 20 weeks.

The immunohistological analysis of transverse TB muscle sections showed that the treatment did not modify the quiescent status of the MPCs at the presymptomatic stage of the disease (Fig. 52A, B). Conversely, at 20 weeks, the full switch of the recruited leucocytes toward the anti-inflammatory phenotype promoted the TB regeneration in treated mice, as demonstrated by the increased percentage of differentiating $Pax7^{-}/MyoD^{+}$ satellite cells compared with the control group (Empty: 10.5±3.6%; MCP1: 19.5±2.3%) (Fig. 52A, C).

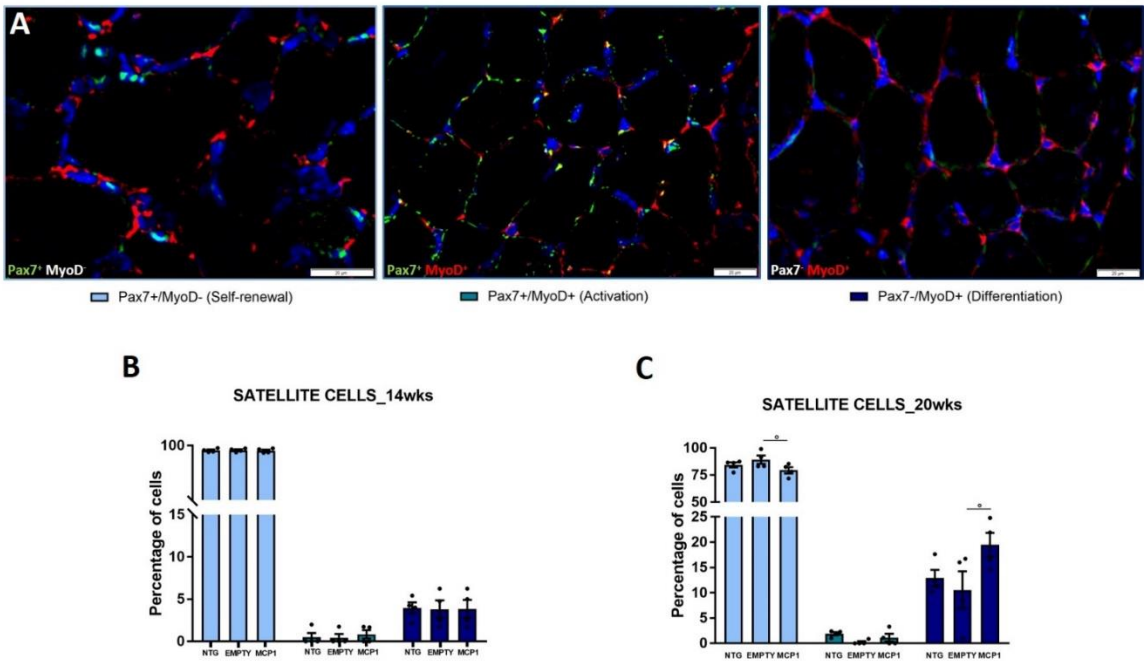

Figure 52: *(A) Confocal micrographs of coronal sections of triceps brachii muscle of ALS mice and non-transgenic (Ntg) littermates stained with Pax7 (red), MyoD (green) and DAPI (blue). Imaging analysis did not show any difference in the myogenic program of satellite cells between the three experimental groups at 14weeks (B). Conversely, increased differentiation of satellite cells was recorded in treated mice compared with the control group at 20 weeks (C). Scale bar= 20µm. Data are expressed as mean±SEM (n=4 per experimental group). °p<0.05 EMPTY Vs MCP1 by Two-way ANOVA with Tukey post-analysis.*

9.4 ANALYSIS OF THE EFFECT OF MCP1 INDUCTION IN THE CERVICAL SPINAL CORD

9.4.1 CHARACTERISATION OF THE EFFECT OF MCP1 INDUCTION ON THE CERVICAL MOTOR NEURON SURVIVAL

The progressive increase of MCP1 within the CNS is classically known as detrimental in promoting the neuroinflammation (Bose and Cho 2013; Conductier et al., 2010). Nevertheless, robust experimental evidence indicates that the chemokine also possesses pleiotropic non-immune

beneficial properties (Semple et al., 2010b; Papa et al., 2018; Locatelli et al., 2012; Chintawar et al., 2009).

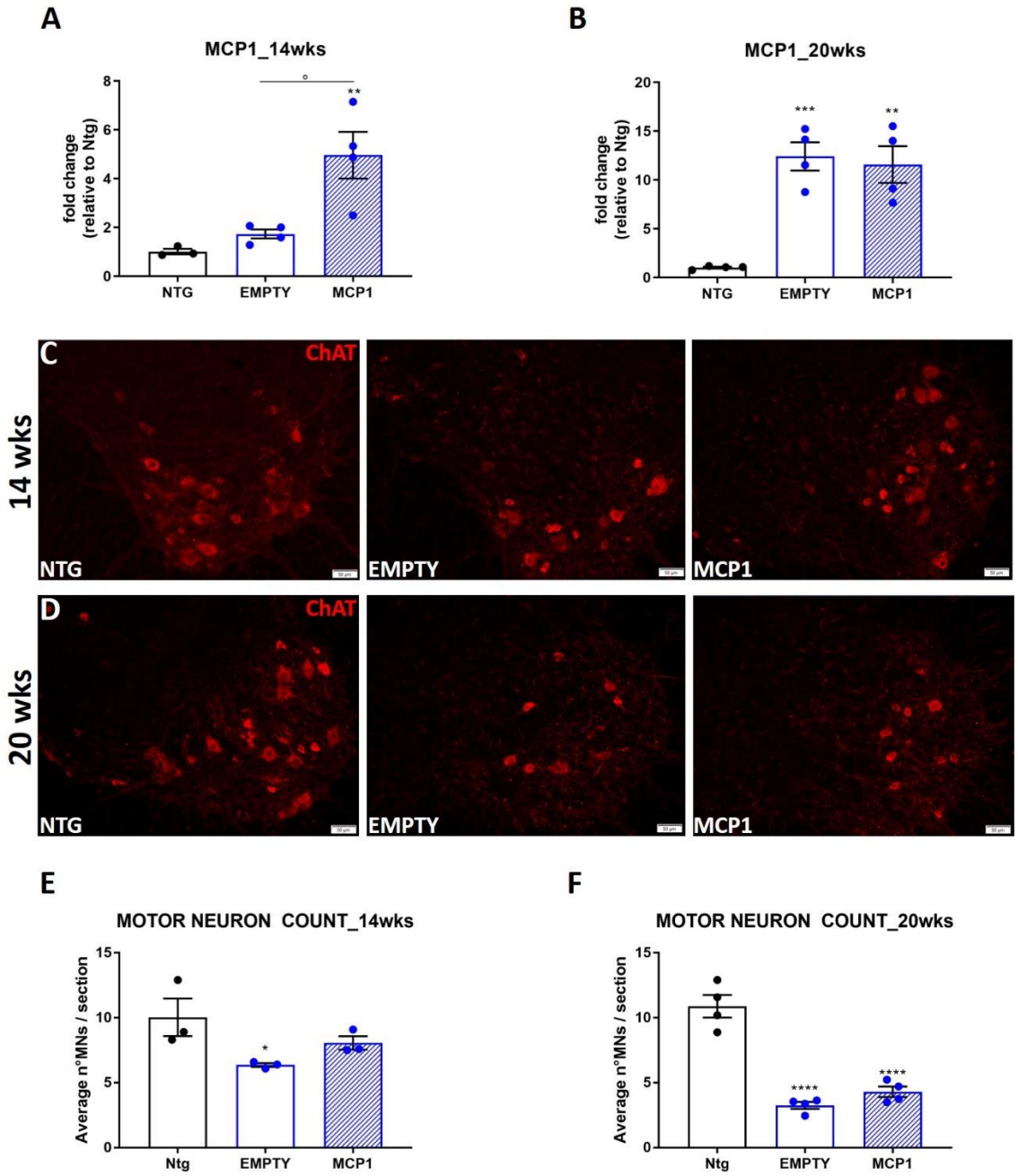

Figure 53: *(A, B) Real-time PCR analysis of MCP1 transcript in the cervical spinal cord of ALS mice and non-transgenic (Ntg) littermates at the presymptomatic (A) and symptomatic (B) stage of the disease. The gene expression analysis revealed a significant upregulation of the chemokine in treated mice compared with the control group at the 14 weeks but not at 20 weeks. Data are normalised to βactin and expressed as mean±SEM (14 weeks: n=3 Ntg, n=4 SOD1^{G93A} Empty or MCP1; 20 weeks n=4 per group). (C, D) Confocal micrographs of coronal sections of the cervical spinal cord of ALS mice and non-transgenic (Ntg) littermates stained with choline acetyltransferase (ChAT) at the presymptomatic (C) and symptomatic stage of the disease (D). Cervical motor neuron count (MN Area > 400μm^2) at 14 weeks (E) and 20 weeks (F). Scale bar= 50μm. Data are expressed as mean±SEM (14 weeks: n=3 per experimental group; 20 weeks: n=4 per experimental group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

The data collected showed that the specific induction of MCP1 in the lower motor unit of C57 SOD1^{G93A} mice significantly reduced the lumbar MNs loss at both time points considered in this study (Figs. 34F and 45B). Therefore, we verified the extent of chemokine induction in the cervical segment of the spinal cord at both the presymptomatic and symptomatic stage of the disease and whether the induction of MCP1 was efficient to protect the cervical MNs from degeneration. The gene expression analysis showed a significant upregulation of the MCP1 transcript in the cervical segment of the spinal cord of treated mice compared with the control groups at the pre-symptomatic stage of the disease (Fig. 53A). However, no difference was recorded between the two groups of ALS mice at the symptomatic disease stage (Fig. 53B).

Suitably, the histological analysis performed at 14 weeks showed a trend in the reduction of the MN death following MCP1 induction, although not significant compared with the scAAV9(empty) treated mice (MN nr Empty: 6.36±0.14, MN nr MCP1: 8.06±0.51) (Fig. 53C, E). However, this protective trend was lost with the disease progression as we did not record any significant difference in the neurodegenerative phenomenon between the two groups of ALS mice at the symptomatic stage of the disease (MN nr Empty: 3.25±0.27, MN nr MCP1: 4.29±0.40) (Fig. 53D, F).

9.4.2 ANALYSIS OF THE GLIA CELLS ACTIVATION AND THE INFLAMMATORY MILIEU IN THE CERVICAL SPINAL CORD

The cervical and lumbar segment of the spinal cord of ALS mice showed a different modulation of the inflammation during the disease progression (Beers et al. 2011b). We found that the induction of MCP1 within the lower motor units of C57 SOD1^{G93A} mice was efficient in modulating the neuroinflammatory phenomenon within the lumbar spinal cord at both presymptomatic and symptomatic disease stage (Figs. 35, 36, 45, 46). Therefore, we assessed the extent of the glial cells activation and the resulting inflammation in the cervical spinal cord of ALS mice upon MCP1 induction.

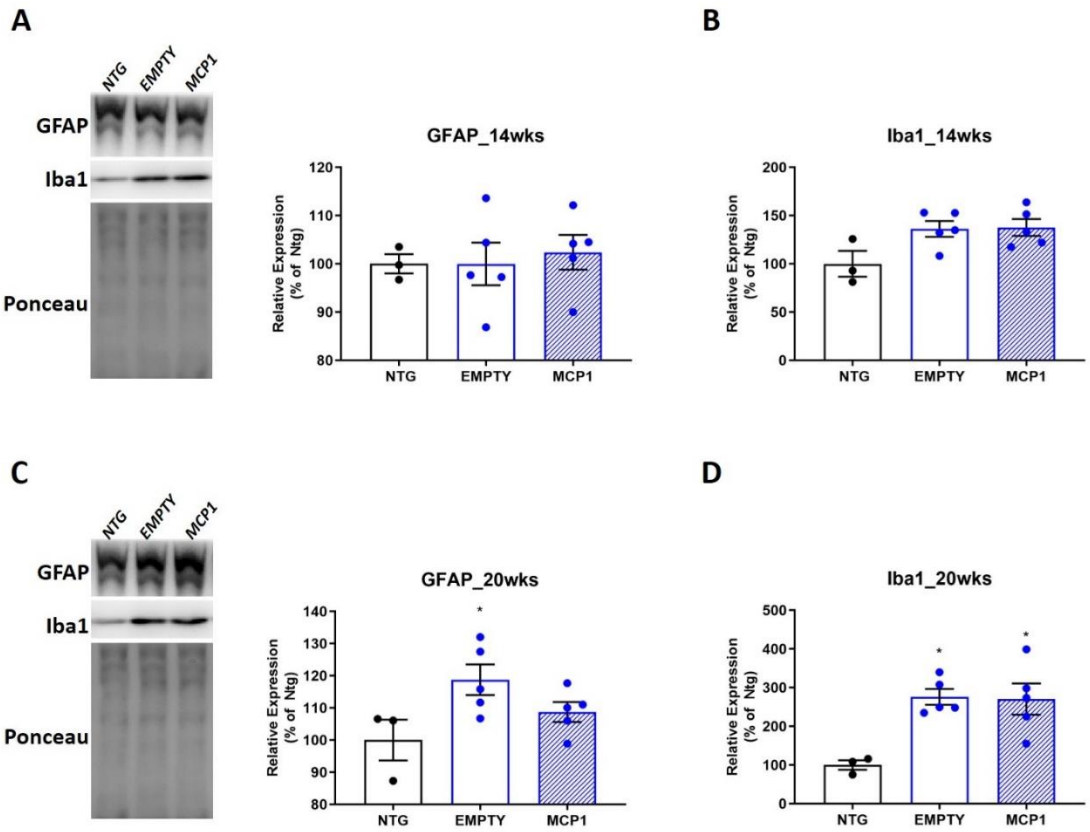

Figure 54: *(A, D) Immunoblot analysis of glial fibrillary acidic protein (GFAP) and ionised calcium-binding adapter molecule 1 (Iba1) performed in cervical spinal cord extracts from ALS mice and non-transgenic (Ntg) littermates at the pre-symptomatic (A, B) and symptomatic (C, D) stage of the disease. The densitometric analysis did not show any difference in the expression of GFAP and Iba1 between the three experimental groups at 14 weeks (A, B). Conversely, a reduced astrocytosis (C) but not microglia proliferation (D) was observed in treated mice compared with the control group at 20 weeks. Data are expressed as mean±SEM (n=3 Ntg; n=5 $SOD1^{G93A}$ Empty or MCP1). *p<0.05 Ntg Vs EMPTY or MCP1 by ANOVA with Tukey's post-analysis.*

The data collected showed that the scAAV9_MCP1 injection modified neither the astrocytes activation nor the microglia proliferation in the cervical spinal cord at 14 weeks (Fig. 54A, B). Conversely, at the symptomatic stage of the disease, we recorded a reduced astrocytosis upon MCP1 induction (Fig. 54C). However, no difference was observed in the extent of microglia proliferation between the two groups of ALS mice (Fig. 54D).

Moreover, in line with the trend in the cervical MN protection observed at the pre-symptomatic stage of the disease, Arginase 1 was significantly upregulated in treated mice compared with the control group at 14 weeks but not at 20 weeks (Fig 55A, E). However, upon MCP1 induction, the transcription level of IL4 resulted upregulated at both time points, although not significantly compared with the scAAV9(empty) treated mice (Fig. 55B, F). Nevertheless, no difference in the

expression of the inflammatory markers gp91^{PHOX} and IL1β was observed between the two groups of ALS mice at both time points (Fig. 55C, D, G, H).

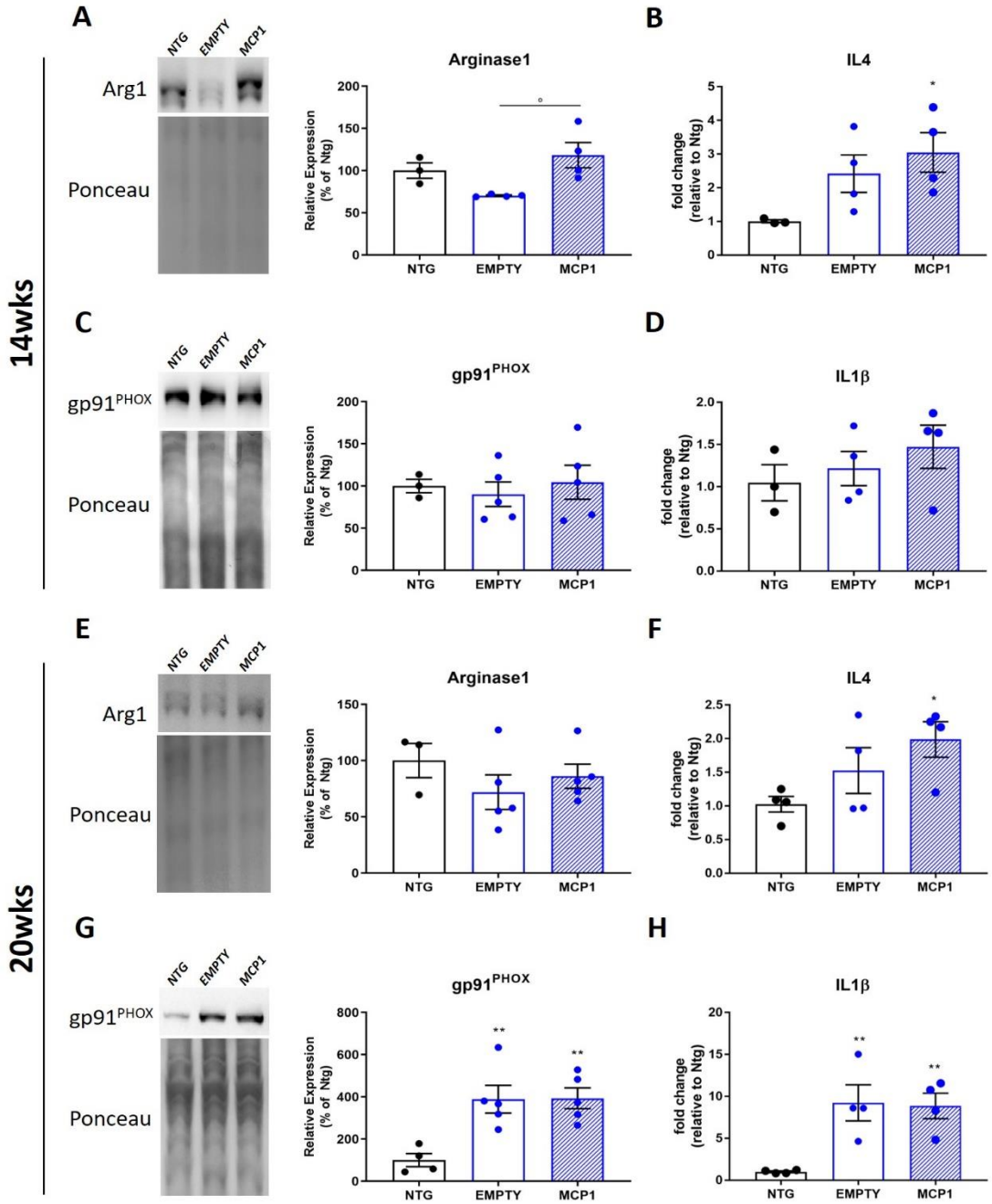

Figure 55: *Representative immunoblot images of arginase 1 (Arg1) and the NADPH oxidase subunit (gp91^{PHOX}) in cervical spinal cord extracts from ALS mice and non-transgenic (Ntg) littermates at the pre-symptomatic (A, C) and symptomatic (E, G) stage of the disease. The densitometric analysis showed an increased expression of Arg1 in treated mice compared with the control group at 14weeks (A) but not at 20 weeks (E). However, the MCP1 induction did not modify the gp91^{PHOX} expression at both time points (C, G). Data are expressed as mean±SEM (14wks: n=3 Ntg; n=4 SOD1^{G93A} Empty or MCP1; 20wks: n=3 Ntg, n=5 SOD1^{G93A} Empty or MCP1). Real-time PCR analysis of Interleukin 4 (IL4) and Interleukin 1ϐ (IL1ϐ) transcripts in the cervical spinal cord of ALS mice and Ntg littermates at the pre-symptomatic (B, D) and symptomatic (F, H) stage of the disease. The gene expression analysis revealed a significant upregulation of IL4 in following MCP1 induction compared with the Ntg littermates, but not scAAV9(empty) treated mice at both time points (B, F). However, no difference in the IL1ϐ transcription was recorded between the two groups of ALS mice at both 14 (D) and 20 weeks (H). Data are normalised to ϐactin and expressed as mean±SEM. (14wks: n=3 Ntg; n=4 SOD1^{G93A} Empty or MCP1; 20wks: n=4 per group). *p<0.05, **p<0.01 Ntg Vs EMPTY or MCP1; °p<0.05 EMPTY Vs MCP1 by ANOVA with Tukey's post-analysis.*

9.5 DISCUSSION

The characterisation of mSOD1 rodent model reported that mice first developed hindlimb tremors, then progressive hindlimb weakness with rapidly deteriorating gates, which eventually culminated in paralysis of one or both hindlimbs (Gurney et al., 1994; Bendotti and Carrì 2004). Forelimb weakness occurred later if at all in disease (Bruijn et al., 1997; Schäfer and Hermans 2011), suggesting a different susceptibility of the upper motor units in the $SOD1^{G93A}$ ALS murine model.

Despite the ascending paralysis is a well-recognised clinical feature of mSOD1 mice, few studies have been aimed at the analysis of the temporal and regional pattern of degeneration of the upper compared with the lower motor units in ALS mice (Capitanio et al., 2012; Beers et al., 2011b; Clark et al., 2016; Nardo et al., 2018). Therefore, the mechanisms underlying the delayed degeneration of the forelimb motor units in mSOD1 mice are still unknown.

The intramuscular injection of the scAAV9_MCP1 prevents the degeneration of the forelimb skeletal muscles of $SOD1^{G93A}$ mice anticipating the peripheral immune response

In line with previous evidence (Clark et al., 2016), our results showed that that the degeneration of the forepaw skeletal muscles is an early event in ALS pathogenic cascade, which already occurs even several weeks before the appearance of motor symptoms in $SOD1^{G93A}$ mice. Indeed, at 14 weeks, the TB muscle of ALS mice was significantly atrophied and denervated compared with the non-transgenic littermates. Nevertheless, during this stage, ALS mice did not recruit immune cells as a mechanism to hamper the degenerative cascade occurring within the skeletal muscles (Ceafalan et al., 2018; Yang and Hu 2018). However, the recent literature described an inductive mechanism of the M1 or M2-polarised immune cells on the muscular progenitor cells, promoting their proliferation and differentiation respectively to accomplish the muscle regeneration (Yang and Hu 2018; Tidball 2017).

Here, we confirm this evidence showing that the anticipation of the inflammatory response through the MCP1-mediated immune cells recruitment protected the TB muscle of $SOD1^{G93A}$ mice from the denervation atrophy since the early stage of the disease. Notably, the significant upregulation of IGF1 recorded at 14 weeks suggested that the scAAV9_MCP1 injection also anticipated the switch

of the infiltrated leucocytes toward the anti-inflammatory phenotype (Tonkin et al., 2015). Accordingly, at the symptomatic stage of the disease, the reduced inflammation in the TB muscle of treated mice compared with the control group was recorded. Specifically, the establishment of the anti-inflammatory milieu within the forepaw muscle of treated mice promoted the differentiation of the satellite cells, thus resulting in the preservation of the tissues from the denervation atrophy.

The intramuscular injection of the scAAV9_MCP1 exerts a protective effect in the cervical spinal cord of SOD1^{G93A} mice at the early stage of the disease

MCP1 is a well-known pro-inflammatory neurotoxic factor produced by activated microglia (Sargsyan et al., 2009; Henkel et al., 2006; Conductier et al., 2010). However, recent evidence suggested a pleiotropic protective role of MCP1 within the CNS unrelated to its chemotactic function (Madrigal and Caso 2014; Papa et al., 2018; Locatelli et al., 2012).

Accordingly, the data herein collected showed that the specific induction of MCP1 within the MN soma partially preserve cervical MNs from degeneration at 14 weeks, increasing the expression of anti-inflammatory factors thus prolonging the so-called stable phase of the disease in mSOD1 mice (Henkel et al., 2009; Beers et al., 2011a). Although small in size, the neuroprotective worthiness of MCP1 was detectable early in the disease, when, as demonstrated by the negligible MN loss and glial cells activation, the cervical spinal cord segment of SOD1^{G93A} mice is almost spare from the degenerative phenomenon (Beers et al., 2011b).

Conversely, at 20 weeks, we did not record any significant increase in the chemokine transcription within the cervical spinal cord of treated mice compared with the control group. This might derive from the progressive MCP1 production by activated microglia cells of mSOD1 mice (Sargsyan et al., 2009; Butovsky et al., 2012), as previously observed in the lumbar segment of the spinal cord of SOD1^{G93A} mice (Fig. 33B). However, the neuroprotective effect mediated by MCP1 was not maintained until the symptomatic stage of the disease. Nevertheless, we recorded an increased transcription of the anti-inflammatory IL4 cytokine in the CNS of treated mice compared with the

control group, which might be responsible for the reduced activation of astrocytes upon MCP1 induction (Brodie et al., 1998; Hu et al., 1995).

In conclusion, the data collected showed that the early induction of MCP1 alongside the upper motor units anticipated the physiologic immune response within the TB muscle of ALS mice, delaying the denervation atrophy. Furthermore, thanks to its pleiotropic neuroprotective activity, the chemokine induction reduced the neuroinflammatory phenomenon within the cervical spinal cord preventing the MN death at the early stage of the disease.

SUMMARY OF RESULTS

Chapter X

The results obtained in this project are summarised in the table below

RATIO MCP1 Vs EMPTY				
Tissue	***Parameter***	C57SOD1^{G93A}		129SvSOD1^{G93A}
		14 weeks	20weeks	17 weeks
TA muscle	MCP1	+182	+180	+11
	Wasting	-0.5	nc	nc
	AChRγ	-2	nc	-2.5
	NCAM	nc	nc	nc
	CD68^{+} cells (IHC)	+2	nc	+1.8
	CD8^{+} cells (mRNA)	nc	nc	+13
	CD4^{+} cells (mRNA)	nc	nc	+7.8
	FoxP3^{+} cells (mRNA)	+5.4	nc	+4.8
	TNFα	-2	nc	+2.3
	gp91^{PHOX}	/	-1.6	+0.7
	IGF1	-0.5	-2.8	-2.4
	Arginase1	+2	nc	nc
	Pax7	nc	nc	nc
	MyoG	+2.2	nc	nc
	MyoD	+1.5	/	/
	Neutrophils	-4.3	/	/
	SIRT1	+1.5	/	/
	% SDH^{+} muscle fibres	-1.1	/	/
	% Pax7^{+}/MyoD^{-} cells	-0.1	/	/
	% Pax7^{+}/MyoD^{+} cells	nc	/	/
	% Pax7^{-}/MyoD^{+} cells	+5	/	/
	Centralized myonuclei	+1.7	/	/
Sciatic Nerve	MCP1	nc	nc	+1.7
	CD68^{+} cells (mRNA)	nc	-1.3	nc
	CD8^{+} cells (mRNA)	nc	nc	+2.2
	TNFα	nc	-1.5	nc
	NF200	/	+2.3	nc
	β importin	/	nc	nc
	p75^{NTR}	nc	+1.5	nc
	MBP	/	+3	nc
Lumbar SC	MCP1	+1.7	nc	nc
	MN nr	+1.4	+1.7	nc
	GFAP	nc	nc	nc
	Iba1	nc	nc	nc
	CD68 (mRNA)	/	nc	nc
	Arginase1	+2.8	nc	nc
	gp91^{PHOX}	nc	nc	nc
	IL1β	nc	-1.7	nc
	IL4	+2.1	/	/

RATIO MCP1 Vs EMPTY				
Tissue	**Parameter**	C57SOD1^{G93A}		129SvSOD1^{G93A}
		14 weeks	20weeks	17 weeks
TB muscle	MCP1	+12	+7.5	+40
	Wasting	-7.7	-1.3	nc
	AChRγ	nc	-2	+2
	NCAM	nc	-2	nc
	CD68^{+}macrophages (IHC)	+2.3	+1.3	/
	CD8^{+} cells (mRNA)	+3.8	nc	/
	CD4^{+} cells (mRNA)	nc	nc	/
	FoxP3^{+} cells (mRNA)	nc	nc	/
	TNFα	+2.7	nc	/
	IGF1	+1.5	nc	/
	Arginase1	nc	+3.2	/
	% Pax7^{+}/MyoD^{-} cells	nc	-1.1	/
	% Pax7^{+}/MyoD^{+} cells	nc	nc	/
	% Pax7^{-}/MyoD^{+} cells	nc	+1.8	/
Cervical SC	MCP1	+2.8	nc	/
	MN nr	nc	nc	/
	GFAP	nc	nc	/
	Iba1	nc	nc	/
	Arginase1	+1.7	nc	/
	gp91^{PHOX}	nc	nc	/
	IL4	nc	nc	/
	IL1β	nc	nc	/

Table 13: *Summary of the results obtained in this project (nc, no change; /, not analysed).*

TA muscle_ The significant MCP1 upregulation recorded in the TA muscle of 14 weeks-old C57 SOD1^{G93A} mice protected the damaged organ from the denervation atrophy anticipating the peripheral immune response. The activation of the inflammatory response in conjunction with the appearance of the first degenerative events allowed its correct management, sustaining the phenotypic switch of the infiltrated leucocytes toward the M2 pro-regenerative phenotype and the eventual activation of the myogenic programme. However, the strength of the MCP1-mediated boosting of the immune response was not sufficient to protect the skeletal muscle until the symptomatic stage of the disease. Nevertheless, the M2 polarisation of the inflammatory milieu was still detectable in the hid paw muscles of 20 weeks-old C57 SOD1^{G93A} mice.

Conversely, the significant MCP1 upregulation recorded in the TA muscle of 129Sv SOD1^{G93A} mice translated in a delayed activation of the immune response that exacerbated the inflammatory phenomenon within the peripheral compartment.

Sciatic Nerve_ The data collected in the 129Sv SOD1^{G93A} mice confirmed that the delayed activation of the inflammatory response is not enough to protect motor axons from degeneration. Conversely, C57 SOD1^{G93A} mice's better capability to manage the inflammatory response (boosted by MCP1) translated into PNS preservation until the advanced disease stage. Notably, our data confirmed the previous evidence (Chiu et al. 2009; Kano et al. 2012) indicating that the sciatic nerve of ALS mice is not damaged enough to require the activation of the inflammatory response at the pre-symptomatic stage of the disease.

Lumbar SC_ The data collected showed that the novel immune unrelated capability of MCP1 was able to extend the so-called stable phase of the neuroinflammatory phenomenon in C57 SOD1^{G93A} mice thus preserving MNs from the degeneration until the advanced disease stage. However, the neuroprotective effect mediated by MCP1 was no detectable in the CNS of 129Sv SOD1^{G93A} mice.

TB muscle_ The early MCP1 induction within a body compartment belatedly affected by the ALS degenerative phenomenon allowed the prompt activation of the inflammatory response and its correct management (M1 -> M2 phenotypic switch) resulting in the TB muscle preservation until the symptomatic stage. Conversely, the scAAV9_MCP1 injection did not preserve the forepaw muscle of 129Sv SOD1^{G93A} mice at the symptomatic disease stage.

Cervical SC_ The data collected confirmed the ability of MCP1 at extending the initial M2-polarised phase of the neuroinflammatory response in the CNS of ALS mice, albeit without protecting cervical MNs significantly. Surprisingly, the neuroprotective effect of MCP1 was lost at the advanced stage of the disease.

GENERAL DISCUSSION and CONCLUSIONS

Chapter XI

GENERAL DISCUSSION and CONCLUSIONS

In this study, we examined the involvement of the MCP1-mediated axis in governing the speed of the disease progression of two ALS models characterised by remarkable differences in the clinical phenotype.

Our observations revealed that the induction of MCP1 in the motor unit of slow progressing $SOD1^{G93A}$ mice ameliorated the clinical phenotype anticipating the recruitment and phenotypic switch of leucocytes within the peripheral compartment, hence sustaining the myogenic programme and nerve regeneration. Conversely, in fast progressing $SOD1^{G93A}$ mice, the treatment exacerbated the inflammatory response resulting in the worsening of the motor ability. Besides, we found that the characterisation of the immune response fingerprint in the skeletal muscle of ALS patients might be a prognostic adjunct useful for a precise stratification in clinical practice.

Intriguingly, our data showed a role for this chemokine in the modulation of the neuroinflammation in the CNS of $SOD1^{G93A}$, with the overall effect of preventing MNs degeneration in the slow progressing but not fast progressing ALS mice.

We recently reported a different activation of MCP1 within MN soma and peripheral compartment of the two $SOD1^{G93A}$ models (Nardo et al. 2013; Nardo et al., 2016b). Notably, our studies revealed that, despite the same extent of MN loss during disease progression (Marino et al., 2015), fast progressing mice showed earlier muscle denervation and motor axon deterioration that correlates with a reduced immune cells (i.e. macrophages and T lymphocytes) infiltration in the peripheral compartment compared with slow progressing ALS mice (Nardo et al., 2016b; Vallarola et al., 2018). We speculated that this defective immune response underpinned the higher peripheral degeneration and the faster disease progression of 129Sv $SOD1^{G93A}$.

This evidence put MCP1, and the eventual peripheral immune cell recruitment, forward as a discriminating factor of the different speed in the disease progression recorded in the two ALS models.

Several lines of evidence indicate a pivotal role of the MCP1-mediated axis in orchestrating the nerve (Stratton et al., 2020; Tofaris et al., 2002; Kwon et al., 2015) and muscle (Shireman et al.,

2007; Lu et al., 2011a; Martinez et al., 2010) regeneration upon damage. Moreover, a pleiotropic neuroprotective activity of the chemokine has been recorded within the injured CNS (Locatelli et al., 2012; Papa et al., 2018).

With this study, the therapeutic potential of the chemokine has been assessed in the two ALS models through the injection of a self-complementary AdenoAssociated Virus serotype 9 engineered with the murine sequence of the *MCP1* gene (scAAV9_MCP1).

Our data revealed that a single injection of the scAAV9_MCP1 in the hind limb and forelimb skeletal muscles of pre-symptomatic (8 weeks-old) 129Sv and C57 SOD1^{G93A} mice was sufficient to transduce the upper and lower motor units in ALS mice. Notably, the experimental protocol herein applied demonstrated its efficiency at inducing the chemokine several weeks after the scAAV9_MCP1 injection (until the symptomatic disease stage: 17 weeks, 129Sv SOD1^{G93A}; 20 weeks, C57 SOD1^{G93A}).

An equipped immune response within the peripheral compartment is fundamental to counteract the ALS progression in SOD1^{G93A} mice

The data obtained by the experimental work detailed in this Thesis indicate that, upon scAAV9_MCP1 injection, the chemokine was upregulated with the same extent in the tibialis anterior (TA) muscle of the two strains of SOD1^{G93A} mice. However, this translated in the amelioration of the clinical phenotype in the slow progressing but not fast progressing ALS mice.

The opposite effect recorded arguably stemmed from a different immune environment developed by the two ALS models within the peripheral compartment upon the scAAV9_MCP1 injection. Indeed, we previously demonstrated that 129Sv SOD1^{G93A} mice are less prone than C57 SOD1^{G93A} mice at activating an efficient immune response within the peripheral compartment at the disease onset (Nardo et al., 2016b; Vallarola et al., 2018). The data collected at the symptomatic stage of the disease endorsed our findings, showing that the immune responsiveness of C57 SOD1^{G93A} mice was finely regulated and, unexpectedly, not further increased by MCP1 induction. Conversely, 129Sv SOD1^{G93A} mice required an exogenous boosting (i.e. MCP1 induction) to elicit the peripheral immune response within the damaged muscle.

The better ability of slow progressing ALS mice in triggering an earlier immune response allowed to properly exploit the chemokine induction hastening the macrophages and lymphocytes recruitment within muscles in the early disease stage, as demonstrated by the data collected six weeks after the scAAV9_MCP1 injection. The activation of the immune response in conjunction with the emergence of the first muscle degenerative events proved to be crucial to allow the phenotypic switch (M1->M2) of the recruited macrophages thus creating a permissive milieu for tissue regeneration (Yang and Hu 2018; Howard et al., 2020; Musarò 2014). Notwithstanding the hindlimb skeletal muscles were significantly damaged at the symptomatic stage, the last glimpse of the protective effect exerted by the early MCP1-mediated immune response was still detectable. Indeed, at 20 weeks, the cytoarchitecture and the myelin wrapping around motor axons were maintained in the scAAV9_MCP1 treated mice. Notably, the PNS preservation recorded upon MCP1 induction might be coupled to the capability of the chemokine at promoting the axonal outgrowth (Locatelli et al., 2012), which is not mediated by its chemotactic power. Regardless, the PNS protection lighted the recruitment of the immune cells, resulting in the reduced toxic inflammation within the sciatic nerve of the treated mice compared with the control group.

Based on these findings, we could surmise that the activation of the peripheral immune response early in the disease course delayed and attenuated the dying-back degenerative phenomenon in the slow progressing ALS mice (Dadon-Nachum et al., 2011; Kano, 2012). However, our data do not provide any hint to understand the worsening of the clinical phenotype found in 129Sv SOD1^{G93A} mice upon MCP1 induction. The different clinical outcome recorded in the fast progressing mice could be ascribed to a strain-related deficit in the activation or management (i.e. M1->M2 phenotypic switch) of the immune response (White et al., 2002; Piirsalu et al., 2020). Based on our findings, we could speculate that this deficiency is exacerbated upon MCP1 boosting through the induction of a persistent inflammatory milieu within the periphery of 129Sv SOD1^{G93A} mice, which significantly hampers tissue regeneration (Büttner et al., 2018; Forcina et al., 2020).

The temporal activation of the immune response as a discriminating factor for the successful regeneration of the peripheral compartment in SOD1^{G93A} mice

The analysis of the effect of the scAAV9_MCP1 injection in the slow progressing mice demonstrated the value of a prompt immune response in slowing down the degeneration of the peripheral compartment and thus the speed of disease progression.

The data collected at 14 weeks demonstrated that the MCP1-mediated early increase in leucocytes recruitment and phenotypic switch significantly delayed the TA muscle degeneration promoting and sustaining the myogenic programme (Yang and Hu 2018; Howard et al., 2020; Musarò 2014; Chazaud 2020). The importance of this anticipated immune response in the C57 SOD1^{G93A} treated mice was detectable even at the symptomatic disease stage when the M2 polarisation of the inflammatory milieu was maintained, and a partial effect on the preservation of neuromuscular junctions innervation was observed. Moreover, the early protection of the skeletal muscles coupled to the intrinsic capability of MCP1 at preserving the axons (Locatelli et al., 2012) might have limited the dying-back degeneration of the neuromuscular system in ALS mice. Indeed, the Schwann cell-mediated response was significantly activated, and the cytoarchitecture and the myelin ensheathment maintained in the sciatic nerve during the disease progression.

The protective effect exerted by the early immune response in preserving the skeletal muscle from degeneration was even more pronounced at the TB muscle level. In line with the delayed involvement of the forelimbs in the SOD1^{G93A} mice pathology (Bruijn et al., 1997; Schäfer and Hermans 2011; Bendotti and Carrì 2004), at 14 weeks the TB muscle exhibited ~20% of muscle wasting compared with the ~40% of the TA muscle. The early induction of MCP1 forced and sustained the immune response in conjunction with the hinted damage of the forepaws muscle. Indeed, higher macrophages and lymphocytes infiltration was recorded in the treated mice compared with the control group, resulting in the almost complete preservation of the TB muscle from the denervation atrophy at 14 weeks.

Intriguingly, the MCP1-mediated early leucocytes recruitment was pivotal in promoting the correct management of the immune response, as demonstrated by the M2 fingerprint of macrophages and

the decreased inflammatory response recorded in the forepaw muscle at 20 weeks. Therefore, the permissive environment established resulted in the significant preservation of the TB muscle, which might be the leading responsible for the ameliorated motor ability recorded in the treated mice at 20 weeks in light of the negligible contribution of the hind limbs in the advanced disease stage (Nardo et al., 2018).

Remarkably, our data strongly support the hypothesis that the delayed immune response is a pathological feature of both ALS strains, as demonstrated by the emptiness of the immune-mediated regenerative mechanisms physiologically activated by C57 SOD1^{G93A} mice. Indeed, our analysis showed that at 14 weeks (i.e. ~2 weeks before the motor onset) the TA muscle is significantly affected by the disease, as demonstrated by the marked atrophy and denervation. Unlike the rapid kinetics of leucocytes recruitment, which is pivotal to rescue skeletal muscle upon acute injury (Yang and Hu 2018), ALS mice have just launched their inflammatory response, as demonstrated by the significant infiltration of neutrophils but not macrophages or lymphocytes.

Altogether this evidence suggests that mSOD1 mice have a poorly responsive immune system, which is unable to promptly activate an effective wound healing process in the peripheral compartments. Accordingly, the delayed immune response within significantly compromised body compartments remarkably affected the phenotypic switch of the recruited immune cells. The end-game is a chronic inflammation which is detrimental to the process of tissue healing (Nathan and Ding 2010). Our observations might mirror previous evidence by Kunis *et al.* (2015), who showed that SOD1^{G93A} mice are immunocompromised and that the enhancing of the leucocytes trafficking within CNS resulting in the amelioration of the disease course.

The phenotypic switch of the infiltrated macrophages is pivotal to protect the skeletal muscles of ALS patients from the degeneration

The preliminary data obtained from the analysis of the muscle biopsies collected from fast and slow progressing ALS patients corroborated our preclinical observations, confirming a crucial role for the immune response in governing the degeneration of the peripheral compartment in the disease.

Due to the delay in the ALS diagnosis, especially in patients with the spinal onset (Richards et al., 2020), the data obtained mirror a full-blown disease stage, at which, as previously shown in the two mSOD1 models herein examined, the activation of MCP1 signalling and the extent of infiltrated immune cells seems to be irrelevant to the speed of the disease progression. Indeed, we did not find any correlation between the ΔFS and the muscular activation of the MCP1 pathway or the extent of $Iba1^{+}$ monocytes infiltration.

Nevertheless, the evidence we have obtained highlight the relevance of the inflammatory fingerprint acquired by the recruited immune cells in preventing the skeletal muscle degeneration. Indeed, the M1 and M2 polarization of the infiltrated macrophages was recorded in the muscle of fast and slow progressing ALS patients, respectively. These observations demonstrate the relevance of the phenotypic switch of recruited macrophages in mediating the protection of the skeletal muscle in ALS.

The easy accessibility in the collection of the muscle bioptic samples paves the way for a more in-depth characterisation of the immune muscle profile, even longitudinally in the ALS course. This might be an attractive tool to shed light on the involvement of the peripheral immune response in governing the speed of the disease progression. Moreover, the characterisation of the muscle profile early in the disease might confirm the evidence of the two $SOD1^{G93A}$ strains, hence putting the peripheral immune signatures forward as a prognostic biomarker in ALS. It will be therefore essential to look at a more systemic profile of immunoregulation in ALS, which would be more accessible in the clinical setting and could mirror the changes thus far identified within the affected muscle.

A Novel immune-unrelated pleiotropic role of MCP1 in the CNS of the $SOD1^{G93A}$ mice

The data herein collected confirmed the previous evidence demonstrating that, besides its well-known toxic inflammatory role (Semple et al., 2010b; Madrigal and Caso 2014), MCP1 possesses an indirect (modulating the recruited immune cells inflammatory phenotype) (Matsubara et al., 2015; Kwon et al., 2015; Niemi et al., 2016) and direct (reducing excitotoxicity and promoting axonal outgrowth) (Locatelli et al., 2012; Papa et al., 2018) neuroprotective role in the CNS.

Our observations demonstrate that the specific induction of MCP1 within the MN perikaryon is effective at reducing the neuroinflammatory phenomenon in SOD1^{G93A} mice. Notably, the protective role was not related to the chemotactic activity of MCP1, since considerable observations have demonstrated that the haematogenous monocytes required an exogenous boosting to enter in the CNS of SOD1^{G93A} mice (Kunis et al., 2015; Chiu et al., 2009; Chiot et al., 2020), but rather to its ability to modulate astroglial activation and hence the polarization of the neuroinflammatory milieu (Quinones et al., 2008; Kalehua et al., 2004; Semple et al., 2010a).

Intriguingly, the data collected in the comparative study of the two SOD1^{G93A} strains showed that the treatment was effective at preventing the MNs degeneration in slow progressing but not fast progressing ALS mice. This disparity in neuroprotective effect might have been influenced by the different modulation of the chemokine basally recorded in the two ALS models during disease progression (Nardo et al., 2013). Indeed, the higher physiological activation of MCP1 in C57 SOD1^{G93A} mice in addition to the scAAV9-mediated chemokine induction might have exerted an additive effect resulting in lumbar MN preservation until the advanced disease stage. Conversely, this additive effect was not sufficient to significantly counteract the neurodegenerative phenomenon in the fast progressing mice, arguably due to the weak activation of the chemokine recorded within the MN of the 129Sv SOD1^{G93A} mice since the early stage of the disease.

The neuroprotective effect of the scAAV9-mediated induction of MCP1 was also detectable in the cervical spinal cord of 14 weeks-old slow progressing ALS mice. Nevertheless, the analysis performed at 20 weeks revealed a weak effect of the treatment at modulating the neuroinflammation hence resulting in the lack of difference in the cervical MN survival between the two experimental groups of C57 SOD1^{G93A} mice. This discrepancy might be linked, as previously discussed for the lumbar spinal cord of the two ALS strains, to the different physiological activation of the chemokine within MNs. Therefore, we can suppose that, at 14 weeks, the scAAV9-mediated chemokine induction was sufficient to exert a beneficial effect arguably thanks to the reduced damage of the cervical spinal cord in the early disease stage. Conversely, as the disease progresses, the scAAV9-derived MCP1 induction was not sustained by the endogenous activation of the

chemokine within cervical MNs, thus resulting in the lack of the neuroprotective effect at 20 weeks. However, we have not evidence regarding the modulation of MCP1 by cervical MN; therefore, further analyses are necessary to verify our hypothesis.

In conclusion, in this project we shed light on the involvement of the peripheral immune system in the ALS course. The evidence herein collected confirm the relevance of the mechanisms involved in the wound healing of the peripheral compartment upon an acute injury (e.g. nerve crush/transection, i.m. injection of toxins) (Gaudet et al., 2011; Yang and Hu 2018) in the chronic degenerative cascade of ALS.

Our observations suggest that, although potentially protective, the peripheral immune response is delayed in ALS mice and hence ineffective to sustain the full recovery of the damaged tissues.

Intriguingly, our evidence showed that the inflammatory fingerprint acquired by the recruited immune cells is pivotal in driving a functional regeneration of the peripheral compartment and thus in defining the speed of the ALS progression. In keeping with this, the clinical observations indicate that the characterisation of the immune muscle profile might serve as prognostic adjunct useful for more precise patients stratification in clinical practice.

Finally, according to our original observation, we confirmed the protective role of MCP1 in the neuromuscular system of ALS mice. Moreover, we demonstrated a novel immune-unrelated capability of the chemokine at modulating the inflammatory phenomenon in the CNS, thus preserving MNs from degeneration.

Altogether, these observations nominate the peripheral compartment as a primary target for the development of effective therapeutic interventions in ALS capable of significantly slow down the disease progression. Moreover, the comprehension of the mechanisms underlying the protective role fulfil from MCP1 in the motor unit of mSOD1 mice might provide innovative evidence regarding the contribution of the immune response in ALS.

PERSPECTIVE STUDIES

Chapter XII

PERSPECTIVE STUDIES

ALS is a multisystemic non-cell autonomous disease (Chiot et al. 2019; Moloney et al. 2014). However, the contribution of the immune response in governing the ALS progression is still debated. Moreover, mounting experimental evidence highlights the different role fulfils from the inflammatory response in the CNS compared with the periphery (i.e. nerves and muscles) (Chiu et al. 2009; Dibaj et al. 2011).

In keeping with this, we started from the observation that the higher NMJs denervation and muscle degeneration underlying the faster disease progression of 129Sv SOD1^{G93A} mice resulted from the faint activation of the peripheral immune response compared with the C57 SOD1^{G93A} mice. This evidence highlights the pivotal role of the peripheral compartment in driving the speed of ALS progression and the involvement of the inflammatory response as a fundamental mechanism to counteract its degeneration.

Intriguingly, here we identified the delayed immune cells recruitment within the peripheral compartment as a pathological feature of mSOD1 mice, which can be exacerbated by the intrinsic (background related) immunological profile. Moreover, the data collected upon MCP1 induction, corroborated by the human ALS bioptic material examination, demonstrated the importance of the activation of a functional peripheral immune response but even harder its correct management (i.e. leucocytes phenotypic switch) once triggered.

Therefore, the characterisation of the inflammatory fingerprint of both circulating and infiltrating immune cells might be an attractive tool to correlate the nature and management of the immune response with the degeneration/regeneration of the peripheral compartment and, thus, the speed of ALS progression.

Aimed to fill this knowledge gap, we will:

- ✓ characterise the blood levels and the transcriptomic profile of the "classically" (M1) and "alternatively" (M2) activated monocyte in fast and slow progressing mSOD1 mice during the disease progression. Besides, the more relevant molecular signature obtained from the pre-clinical analysis will be validated in the peripheral

blood mononuclear cells (PBMCs) derived from fast and slow progressing ALS patients.

- ✓ evaluate the immunological fingerprint of macrophages infiltrated within the skeletal muscles of fast and slow progressing mSOD1 mice and ALS patients.
- ✓ characterise the fast and slow progressing mSOD1 mice derived macrophages' responsiveness to pro-inflammatory/anti-inflammatory stimuli and their capability to influence the satellite cells response.

This project will be founded by the Italian Ministry of Health (SG2019 - 12371083).

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